Ecology Of Ecto- And Ericoid Mycorrhizal Systems In Petroleum Hydrocarbon-Contaminated Sub-Boreal Forest Soils

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Dissertation Submitted In Partial Fulfillment Of

The Requirements For The Degree Of

Doctor of Philosophy

in

Natural Resources and Environmental Studies

The University of Northern British Columbia

December 2008

Your file Votre référence
ISBN: 978-0-494-48846-1
Our file Notre référence
ISBN: 978-0-494-48846-1

Abstract

The impacts of petroleum hydrocarbon (PHC) contaminants in northern forest soils are not well understood from either eco-toxicological or microbial ecological perspectives. The purpose of this research was to examine interactions between PHCs and ecto- (ECM) and ericoid (ERM) mycorrhizal communities at the rhizosphere scale, where microbial activities underpin processes such as decomposition, carbon and nutrient cycling, and primary production at landscape scales. Several methodological approaches were simultaneously used to assess changes in physical, chemical and biological properties of plant-soil systems treated with ecologically relevant concentrations (i.e. ~7-22 tonnes ha^{-1}) of PHCs. From microscopy and community fingerprinting (LH-PCR) studies, we found few differences in community structure attributable to PHC contamination. PHC treatment also did not appear to alter broad patterns of C metabolism for either bacterial (CLPP) or ECM fungal (laccase assay) communities. Habitat changes, which generally included increased C:N ratios, slightly more acidic pH, and hydrophobicity or water-logging in organic and mineral layers, respectively, did not appear to inhibit microbial communities. Together, these findings point to resilience within intact mycorrhizal systems, mainly due to sorption of PHCs within organic soil layers and protective properties of the mycorrhizosphere habitat. Soil PHC analysis (GC-FID) showed a general decrease in contaminant levels 16 weeks after PHC treatment, indicating an intrinsic capacity for biodegradation within the soil communities. ECMs appeared to play a vital role in this process through provision of habitat and co-substrates for heterotrophic bacterial communities (i.e. mycorrhizosphere effect) and via secretion of laccase, which opens aromatic ring structures for subsequent bacterial attack. These results emphasize the importance of synergistic functions among microbial guilds with

respect to ecological processes. Finally, we found that the spatial patterns of mycorrhizal communities within the rhizosphere depended primarily on properties of the host plant and soil environment. The extent that different properties influenced community structure varied between the three groups of microorganisms. This systems approach addressed fundamental questions in mycorrhizal ecology by considering PHC pollution as a form of environmental disturbance. Conservation of the integrity of mycorrhizal systems in contaminated forest soils may be key for sustainable management in terms of ecosystem resilience and remediation.

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Acknowledgements

I could not have finished this thesis without the support of many people. I especially thank my co-supervisors, Hugues Massicotte and Mike Rutherford, for contributing so much of their time to discussing ideas and data, reading and editing papers, and supporting my emotional break-downs. I also thank my supervisory committee, Bill McGill, Paul Sanborn, Keith Egger, and Shannon Berch, for their valuable contributions to the design, techniques, analysis, and interpretations of this research, as well as insightful comments on earlier drafts of this work. I am additionally indebted to Alex Hawley for helping me to connect the dots.

I thank fellow fungal researchers, Nabla Kennedy, Kei Fujimura, Monika Gorzelak, Brian Pickles, and Jocelyn Campbell, for friendship and good advice in multivariate statistics, science, and life. I am particularly grateful to Linda Tackaberry for her sympathetic ear, regular coffee breaks, editorial suggestions, and guidance through exhausting administrative mazes. My gratitude, also, to the people who provided materials or helped with technical aspects of this work: Dawn Stubley (Tree Seed Center, Surrey), Barb Rayment (Birch Creek Nursery, Prince George), Ralph Alms (Husky Inc., Prince George), Steve Storch and John Orlowsky (Enhanced Forestry Lab), Quanji Wu (PHC analysis), Anna Scarpino, Allen Esler, and Clive Dawson (soil analysis), and Dana O'Bryan and Mark Thompson (DNA analysis). I am also very grateful for my extremely understanding family and friends for still talking to me after several years of sporadic contact. Special thanks to Andy Kendrick, who has vicariously endured eight years of grad school in eight years of marriage, and Misha, for hanging in there.

Finally, this research would not have been possible without financial support provided by the Natural Sciences and Engineering Research Council of Canada through grants to Hugues Massicotte and Mike Rutherford and a PGS-B scholarship to me. I am also very grateful to the University of Northern British Columbia for providing laboratory facilities and travel grants for conferences.

Introduction

The ubiquity and enormous biomass of mycorrhizal systems (i.e. plant-fungal symbioses and bacterial communities associated with the mycorrhizosphere) in northern forest soils implies a key role in forest ecosystem processes. Although recent studies have revealed enormous taxonomic and genetic diversity of mycorrhizal communities associated with certain plants in some ecosystems, the specific activities of individual or groups of taxa and their contributions to processes across heterogeneous landscapes are not well understood. The complexity of the soil environment and the multifunctional nature of many microorganisms have made it difficult to predict how ecosystems may respond to environmental disturbances such as soil contamination with crude oil. Very little is currently known regarding the fate and impacts of petroleum hydrocarbon (PHC) contaminants in forest soil ecosystems.

The purpose of this research was to examine interactions between PHCs and mycorrhizal communities in plant-soil systems, where different guilds of microorganisms interact within the larger trophic group tightly linked to decomposition, carbon and nutrient cycling, and primary production. The microbial groups assessed in the current study include ecto- (ECM) and ericoid (ERM) mycorrhizal fungi and associated bacterial communities. A combination of methodological approaches were simultaneously used to gain a better understanding of the physical, chemical and biological changes that occurred in the rhizosphere following contamination with ecologically relevant (i.e. equivalent to several tonnes per hectare) levels of oil. The specific objectives of each study are summarized in the following paragraphs for each of the five thesis chapters. All chapters are written in the first person plural in recognition of contributions of others to this work.

Chapter 1 is a review of literature from several different fields, including botany, mycology, microbial ecology, soil science, and environmental toxicology. This synthesis is presented from the perspective of mycorrhizal systems, which represent the structural and functional interface between decomposition and primary production processes in northern forest ecosystems. It is an attempt to form linkages between the often disparate bits of knowledge that contribute to the current understanding of the fate of PHCs in boreal forest soils in both ecological and toxicological contexts. The literature review helped to inform the direction of research presented in the subsequent chapters. This paper was published in Biological Reviews (Robertson *et al.*, 2007).

A fundamental question is addressed in Chapter 2: does PHC contamination of forest soils negatively impact established (or establishing) mycorrhizal systems? We hypothesized that the structure (composition) and diversity (richness and relative abundance) of ECM/ ERM fungal and associated bacterial communities would be altered in response to PHC treatment, either via soil habitat changes, chemical toxicity, or altered C or nutrient regimes. We further expected that systems with different host plant (e.g. root structure and exudation patterns) or soil properties (e.g. previous PHC contamination history or high lignin content) would contain mycorrhizal communities adapted to PHC contamination conditions to varying extents. Using a bioassay (single- and double-plant systems established in reconstructed forest soil layers in pots), morphological and molecular (LH-PCR) techniques were used to assess changes in mycorrhizal community structure in response to different PHC concentrations over 16 weeks. Laccase assays were also conducted to assess changes in

enzyme activity associated with PHC treatment. Results of these assessments are discussed in terms of ecological integrity and remediation/ restoration.

Chapter 3 focuses on the indirect role of the mycorrhizosphere in the biodegradation process (i.e. provision of habitat for consortia of bacteria involved in metabolizing PHCs) and examines effects of PHCs on structure and metabolic function of bacterial communities. We hypothesized that indigenous bacterial communities would have the capacity for PHC biodegradation, at least for the smaller fraction PHCs, and that this capacity would be enhanced in the presence of mycorrhizal root systems that provide C substrates (exudates) for co-metabolism. In addition, we expected that the mycorrhizosphere would provide some protection of bacterial communities from potentially toxic effects of some PHC chemicals. Single-, double- and no-plant systems were treated with the highest PHC level (i.e. 219 mg cm^{-2}) tested previously. GC-FID analysis was used to determine concentration of PHCs (in 4 fractions) in soil layers at 1 and 16 weeks; soil layers were analyzed for C:N ratio and pH. Genotypic profiles of root-associated bacterial communities were assessed using LH-PCR analysis; metabolic profiles were based on C substrate use after 7 weeks. These results are discussed in terms of the mycorrhizosphere effect, functional redundancy, and syntrophic biodegradation of PHCs.

In Chapter 4, the relative contributions of plant and soil properties to spatial distribution patterns of ecto- (ECM) and ericoid (ERM) mycorrhizal fungi as well as root-associated bacterial communities inhabiting the shared rhizosphere of pine (ECM host) and lingonberry (ERM host) were investigated in PHC-treated and control systems. Community profiles (i.e.

based on the relative abundance of all genotypes) were generated for all root systems using LH-PCR and primers targeting the ITS (fungi) and 16S (bacteria) regions of ribosomal DNA. ECM composition, relative abundance and variation between soil layers were also assessed using light microscopy. Spatial distribution patterns of the three groups of microorganisms (i.e. ECM and ERM fungi and associated bacteria) are compared and discussed with respect to habitat and niche considerations in the plant-soil systems.

Final conclusions and considerations for future research are presented in Chapter 5. It is a general discussion along several themes that emerged during analyses in the three data chapters, including the rhizosphere as an ecological unit, system resilience to disturbance, syntrophic biodegradation of PHCs, functional redundancy within and between trophic groups, importance of scale, variation and uncertainty in ecological data, and ecological foundations for sustainable forest management.

Robertson, S.J., McGill, W.B., Massicotte, H.B. and Rutherford, P.M. (2007) Petroleum hydrocarbon contamination in boreal forest soils: a mycorrhizal ecosystems perspective. *Biological Reviews* 82: 213-240.

Chapter 1: Petroleum hydrocarbon contamination in boreal forest soils: a mycorrhizal ecosystems perspective

Abstract

The importance of developing multi-disciplinary approaches to solving problems relating to anthropogenic pollution is now clearly appreciated by the scientific community, and this is especially evident in boreal ecosystems exposed to escalating threats of petroleum hydrocarbon (PHC) contamination through expanded natural resource extraction activities. This review aims to synthesize information regarding the fate and behaviour of PHCs in boreal forest soils in both ecological and sustainable management contexts. From this, we hope to evaluate potential management strategies, identify gaps in knowledge and guide future research. Our central premise is that mycorrhizal systems, the ubiquitous root symbiotic fungi and associated food-web communities, occupy the structural and functional interface between decomposition and primary production in northern forest ecosystems (i.e. underpin survival and productivity of the ecosystem as a whole), and, as such, are an appropriate focal point for such a synthesis. We provide pertinent basic information about mycorrhizas, followed by insights into the ecology of ecto- and ericoid mycorrhizal systems. Next, we review the fate and behaviour of PHCs in forest soils, with an emphasis on interactions with mycorrhizal fungi and associated bacteria. Finally, we summarize implications for ecosystem management. Although we have gained tremendous insights into understanding linkages between ecosystem functions and the various aspects of mycorrhizal diversity, very little is known regarding rhizosphere communities in PHC-contaminated soils. This makes it difficult to translate ecological knowledge into environmental management strategies. Further research is required to determine which fungal symbionts are likely to

survive and compete in various ecosystems, whether certain fungal - plant associations gain in ecological importance following contamination events, and how PHC contamination may interfere with processes of nutrient acquisition and exchange and metabolic processes. Research is also needed to assess whether the metabolic capacity for intrinsic decomposition exists in these ecosystems, taking into account ecological variables such as presence of other organisms (and their involvement in syntrophic biodegradation), bioavailability and toxicity of mixtures of PHCs, and physical changes to the soil environment.

Key words: ectomycorrhiza, ericoid mycorrhiza, mycorrhizal ecosystems, boreal forest soils, ecosystem processes, petroleum hydrocarbons, soil pollution, biodegradation, bioremediation.

Introduction

Boreal and sub-boreal forest ecosystems include arctic, sub-arctic and northern mid-latitude forest regions that are dominated by a cold climate and are able to support only a few coniferous and broadleaf tree genera (Burton *et al.*, 2003). Petroleum hydrocarbons (PHCs) are complex mixtures of aliphatic, alicyclic and aromatic compounds (Miller and Herman, 1997; Potter and Simmons, 1998) plus constituents that contain N, S or O in addition to H and C. PHCs may find their way into terrestrial ecosystems by surface spills or leaks from pipelines or storage tanks. The microbial ecology of boreal forest ecosystems, with or without PHCs, is incompletely understood. It is known, however, that symbiotic fungi colonize and extend beyond the roots of dominant plant species, thereby forming an intimately interwoven belowground mycorrhizal system. Mycorrhizal fungi account for most of the microbial biomass in organic soil horizons (Lundström *et al.*, 2000; Dahlberg, 2001). The traditional role of individual symbioses involves the exchange of soil nutrients for carbohydrates fixed through plant photosynthesis (Smith and Read, 1997). Nutrients are obtained from inorganic sources inaccessible to plants or accessible, but more readily obtained, by the mycobiont. However, some mycorrhizal systems appear to possess well-developed saprotrophic capabilities (i.e. oxidative and hydrolytic enzyme systems) that mobilize nutrients from organic sources. Such capabilities may have developed through selection in ecosystems characterized by slow decomposition and retention of nutrients in organic polymers (Hibbett *et al.*, 2000; Burke and Cairney, 2002; Cairney and Meharg, 2002; Read and Perez-Moreno, 2003). Mycorrhizal systems capable of metabolizing exogenous organic compounds therefore may be candidates for use in remediation of soils contaminated with PHCs.

Mycorrhizal fungal mycelia and surrounding soil (i.e. mycorrhizosphere) provide suitable habitats for diverse communities of microorganisms due to increased availability of high-energy metabolic substrates and surfaces for colonization (Sarand *et al.*, 2000; Sen, 2003; Heinonsalo *et al.*, 2004). This enhances bacterial decomposition of plant materials because mycelia provide a path, together with associated water films, through which bacteria can migrate to substrates in micropores. Metabolic synergism between fungal and bacterial members of soil communities ensures that virtually all organic compounds are subject to biotransformation (if available to decomposer organisms) and that nutrients and energy-rich compounds are exchanged between plants and the soil environment *via* mycorrhizal fungal networks (Simard *et al.*, 1997; Read and Perez-Moreno, 2003; Díaz, 2004; Heinonsalo *et al.*, 2004). Consequently, in addition to their direct transformation of organic compounds, mycorrhizal systems may indirectly enhance degradation of PHCs in soil by modifying the structure of associated bacterial communities (Cairney and Meharg, 2002).

Oil extraction, refinement and transportation activities in boreal regions have resulted in surface and near-subsurface soil contamination with PHCs including crude (or synthetic crude) oil, gasoline, diesel and creosote (Kanaly and Harayama, 2000). The current standard against which environmental impacts are evaluated is sustainability (maintenance of ecological integrity) using various ecological indicators as measures. Sustainability requires management strategies for large areas and long periods of time that satisfy diverse environmental, social and economic needs (Burton *et al.*, 2003). The relationship between soil microbial communities and ecosystem processes (e.g. decomposition and

biogeochemical cycling) provides insights into how communities and ecosystems respond to environmental change. Microbial diversity (the variety of taxonomic, genetic and functional characteristics of organisms) helps sustain terrestrial ecosystems by conferring ecosystem stability (the ability to withstand change), resilience (the ability to recover from change) and resistance (the inherent capacity to withstand disturbance) (Andrén and Balandreau, 1999; Tiedje *et al.*, 1999; Nannipieri *et al.*, 2003; Swaminathan, 2003; Fitter *et al.*, 2005). Lower diversity or higher specialization occurs in disturbed soil systems due to: (1) extinction of populations that lack sufficient tolerance to the change imposed, and/or (2) selective enrichment of populations that tolerate or thrive under the new conditions (Díaz, 2004; Hofman *et al.*, 2004). To understand the basis of community differences associated with changes in environmental conditions, it is necessary to integrate the functional properties and environmental requirements or tolerances of communities with processes at an ecosystem level (Bengtsson, 1998; Cairney, 1999; Dahlberg, 2001; Read and Perez-Moreno, 2003).

The potential toxicity of some PHCs to human, plant and animal receptors is used in managing contaminated sites, but the physical, chemical and biological impacts on soil microbial communities are less extensively studied and used (Miller and Herman, 1997; Nicolotti and Egli, 1998). Controlled experiments have provided valuable information regarding the toxicological impacts of chemicals on test organisms, which forms the scientific basis for current remediation standards. In soils, toxicity of PHCs to soil organisms including plants occurs concurrently with physical and chemical changes to the soil habitat following PHC contamination (Tarradellas and Bitton, 1997; Blakely *et al.*, 2002; Trofimov and Rozanova, 2003). Is it possible to separate the effects of chemical toxicity from habitat

changes such as hydrophobicity, lowered redox potential or reduced nutrient supply in PHC-contaminated soils? Are methods available for assessing the fate and behaviour of PHCs in forest soils that include bioavailability and indicators for ecological integrity that also complement measures for plant productivity? In addition, many PHCs are structurally analogous to organic compounds naturally found in the soil environment and appear to be degraded by soil microbial communities using the same biochemical pathways (McGill *et al.*, 1981; Siciliano and Germida, 1998). Can functional aspects of microbial populations and communities (e.g. exocellular enzymes) be manipulated for bioremediation of contaminated soil?

Numerous reviews have addressed various aspects of mycorrhizal systems (e.g. Meharg and Cairney, 2000; Dahlberg, 2001; Burke and Cairney, 2002; Allen *et al.*, 2003; Read and Perez-Moreno, 2003; Fitter *et al.*, 2005) or of PHC behaviour and biodegradation in soil (McGill *et al.*, 1981; Riser-Roberts, 1998; Alexander, 1999, 2000; Prince and Drake, 1999; Díaz, 2004; Stokes *et al.*, 2005; Römbke *et al.*, 2006). How might we advance the understanding of the fate and behaviour of PHCs in boreal forest soils in both ecological and sustainable management contexts? We aim to do so by synthesizing information regarding the interactions between mycorrhizal communities and PHC contaminants in boreal soils. From this, we hope to evaluate potential management strategies, identify gaps in knowledge and guide future research. Our central premise is that mycorrhizal systems occupy the structural and functional interface between decomposition and primary production in northern forest ecosystems and as such are an appropriate focal point for such a synthesis. Information in this synthesis should be useful to professionals ranging from ecologists to

engineers involved in the management and remediation of contaminated boreal forest soils. Our approach is first to provide pertinent basic information about mycorrhizas, followed by insights into the ecology of ecto- and ericoid mycorrhizal systems. Next we review the fate and behaviour of petroleum hydrocarbons in forest soils, with an emphasis on interactions with mycorrhizal fungi and associated bacteria. Finally, we summarize implications for ecosystem management.

Mycorrhizas

Classification and structure

Mycorrhizas are symbioses between plant roots and an array of soil-inhabiting, filamentous fungi. These associations are virtually ubiquitous and generally considered mutualisms (i.e. reciprocally increase the fitness of both partners) as they are based on a bidirectional exchange of nutrients that is essential to the growth and survival of both partners (Smith and Read, 1997; Peterson and Massicotte, 2004; Sapp, 2004). The fungal partner acquires nitrogen (N), phosphorus (P) and other nutrients from the soil environment and exchanges them with the plant partner for photosynthetically derived carbon (C) compounds that fuel fungal metabolism. The structural attributes of mycorrhizas are related to their primary function of nutrient exchange and provide the basis for broad classification into seven currently recognized groups: ectomycorrhizas, ericoid mycorrhizas, ectendomycorrhizas, arbuscular mycorrhizas, arbutoid mycorrhizas, monotropoid mycorrhizas and orchid mycorrhizas (Peterson *et al.*, 2004). In boreal forest ecosystems, most trees typically form ectomycorrhizal (ECM) symbioses, whereas the major constituents of the understorey

vegetation often form arbuscular (AM), ericoid (ERM) or arbutoid (ARM) mycorrhizas. The ECM and ERM groups will be considered herein in the greatest detail.

Ectomycorrhizas, the associations between ECM fungi and the roots of woody plants, are characterized by three structural components: the mantle, the Hartig net and the extraradical mycelium (Smith and Read, 1997). The mantle is a sheath of fungal tissue that covers the highly active tips of the lateral roots of the plant and forms the boundary between the root and the soil environment. Its compact, but also variable, morphological nature provides a buffering capacity that helps to prevent root cell dehydration or penetration by pathogenic organisms (Brundrett, 1991). Fungal cells (hyphae) emanate from the outer mantle as extraradical mycelia and grow into the surrounding soil where they reach micropore areas and absorb nutrients that may otherwise be inaccessible, both physically and biochemically (i.e. enzymatic processing of organic compounds), to roots (Söderström, 1992; Perez-Moreno and Read, 2000). Some ECM fungi also form rhizomorphs, which are thick linear aggregates of hyphae that are specialized for long-distance translocation of nutrients and water (Agerer, 2001). Lipids, phenolic compounds, proteins and polyphosphates may accumulate in the hyphae of the outer mantle, which may also bind heavy metals and thereby prevent their uptake into roots (Peterson *et al.*, 2004). The inner mantle consists of repeatedly branched hyphae, suggesting a role in nutrient exchange such as enabling absorption of glucose and fructose from the root and conversion to fungal sugars (e.g. trehalose, mannitol or glycogen) (Peterson *et al.*, 2004). At the interface of nutrient exchange is a highly branched structure known as the Hartig net, which is formed by multidigitate growth of fungal hyphae between epidermal and cortical cells of the root, and is the probable site for exchange of resources

between symbionts (Peterson *et al.*, 2004). Subtle variations in morphological attributes viewed using light microscopy are often used to distinguish between ECM fungal taxa; development and differentiation of extraradical mycelia may provide predictive features relevant to the ecological classification of ECMs (Agerer, 1987-2002, 2001).

The common feature of plants that form ericoid mycorrhizas is the formation of very fine lateral roots that are composed of a vascular cylinder, one or two rows of cortical cells and an epidermal layer of enlarged cells (Peterson *et al.*, 2004). ERM fungi do not form mantles or Hartig nets, but rather colonize the epidermal cells of these fine roots and develop intracellular hyphal coils that are specialized for nutrient exchange (Peterson *et al.*, 2004). The intracellular fungal symbiont is separated from the plant cytoplasm by a plant-derived membrane, which invaginates to follow fungal growth and coil formation (Perotto *et al.*, 2002). ERM fungal taxa cannot be distinguished by morphological characters using light microscopy. From molecular studies, it appears that ERM roots are composite structures that house multiple fungal symbionts, which implies that epidermal root cells may potentially function as separate units colonized by a variety of fungi (Perotto *et al.*, 2002).

Diversity

Mycorrhizal symbioses have been an important force in evolution (Pirozynski and Malloch, 1975; Blackwell, 2000; Cairney, 2000; Sapp, 2004). Based on reconstructions of evolutionary lineages (phylogenies) from fungal DNA and the fossil record, it is currently accepted that the first mycorrhizal associations were pivotal in allowing plants to colonize the terrestrial environment about 600 million years ago and they form the evolutionary basis

of present plant communities (Pirozynski and Malloch, 1975; Blackwell, 2000). Redecker *et al.* (2000) reported fossilized fungal hyphae and spores found from the Ordovician of Wisconsin (about 460 million years old) that strongly resemble modern Glomerales-like AM fungi. Modern AM fungal species persist in most extant plant species and form a single monophyletic group descended from these first mycorrhizas (Cairney, 2000). The AM group represents four orders (Archaeosporales, Paraglomerales, Diversisporales and Glomerales) of fungi within the phylum Glomeromycota (Smith and Read, 1997).

ECM fungal diversity appears to have arisen about 200 million years ago, corresponding to changes in climate that allowed for colonization of the land with trees and increased organic matter content of some ancient soils (Cairney, 2000). Although ECM plant partners (phytobionts) represent only about 8000 species (mostly in the families Pinaceae, Betulaceae, Fagaceae, Dipterocarpaceae, Salicaceae and Myrtaceae), these species are of global importance because of their disproportionate occupancy and domination of terrestrial ecosystems in boreal, temperate and subtropical forests (Smith and Read, 1997). It has been estimated that 5000-6000 species of fungi (of the sub-divisions Basidiomycotina, Ascomycotina and Zygomycotina) form ECM symbioses (Molina *et al.*, 1992; Horton and Bruns, 2001), but these numbers are expected to rise as more regions are progressively explored in detail (Cairney, 2000). Phylogenetic analyses reveal that ECM fungi have originated from several independent lineages and that symbiosis with plants has been convergently derived (and perhaps lost) many times over millions of years (Hibbett *et al.*, 2000). Some ECM taxa are closely related to, or descended from, wood-rot fungi and some are related to other saprotrophic fungal taxa (Tanesaka *et al.*, 1993; Hibbett *et al.*, 2000).

This variation in the ability to degrade wood may have helped drive fungal speciation to avoid competition between closely related species that would otherwise use the same resources and occupy the same niche (Tanesaka *et al.*, 1993; Bruns, 1995; Martin *et al.*, 2000). The ability to degrade the complex aromatic chemical structures of lignin in wood may also confer an ability to transform similar structures in PHCs.

ERMs evolved about 100 million years ago, as sclerophyllous vegetation (i.e. plants with small, tough foliage and tissues that are rich in lignin and cellulose, but deficient in N and P) emerged in nutrient-poor soils (Cairney, 2000). Many plants of the family Ericaceae (e.g. *Vaccinium, Rhododendron, Gaultheria, Ledum* species) are common components of the understorey vegetation in northern forests and usually form typical ERMs (Vrålstad *et al.*, 2002*b*). In the Southern hemisphere, plant species of the family Epacridaceae form ERMs (Cairney and Ashford, 2002). ERM fungi were thought to belong to the Ascomycotina, of which fungal strains in the *Rhizoscyphus ericae – Scytalidium vaccinii* species complex (Helotiaceae, Helotiales, Ascomycota) are most commonly studied and reported (Vrålstad *et al.*, 2002*a*; Zhang and Zhuang, 2004). In addition, ERM fungi identified as *Oidiodendron* (anamorphs of the ascomycete family Myxotricaceae) as well as a broad range of sterile mycelia with divergent morphologies and unknown identifications have been described (Vrålstad *et al.*, 2002*a*). Recent morphological (clamped hyphae and dolipore septae forming typical ERM coils on *Vaccinium*, *Rhododendron* and *Gaultheria* species) and molecular (rDNA sequences) evidence indicates that some ERM fungi may belong to the Basidiomycotina (Berch *et al.*, 2002; Perotto *et al.*, 2002). It has become increasingly

apparent that a wider spectrum of taxa is involved in the ERM symbiosis than had been previously imagined.

ECM communities appear to consist of large numbers of fungal species (i.e. exhibit high species richness), even within small areas with little heterogeneity in plant communities, soil properties, climate and disturbance patterns (Bruns, 1995; Kranabetter *et al.*, 1999; Taylor *et al.*, 2000; Mah *et al.*, 2001; Robertson *et al.*, 2006). ERM fungal communities also appear to exhibit high richness. For example, Monreal *et al.* (1999) isolated 20 fungi (five of which formed ERM *in vitro*) from sixty segments (each 3 mm long) of fine roots from an 8-cm-long salal (*Gaultheria shallon* Pursh) rhizome. This ERM fungal richness is consistent with other reports of species-rich communities of mycorrhizal and non-mycorrhizal endophytes in individual root systems of other ericaceous [e.g. *Calluna vulgaris* (L.) Hull] and epacridaceous [e.g. *Woollsia pungens* (Cav.) F. Muell.] plants. All groups of ericoid fungi reported globally have been found associated with salal from a single site on Vancouver Island (British Columbia, Canada) and all ERM groups reported on salal have been found associated with other plant species elsewhere in the world (Berch *et al.*, 2002). It is currently hypothesized that sterile mycelia with ERM behaviour represent a heterogeneous group of fungal taxa that are mostly unidentified and appear to include a variety of unculturable mycobionts (Berch *et al.*, 2002; Perotto *et al.*, 2002). High species richness and abundance may represent ecological adaptation to local environmental heterogeneity and is thought to provide forests with a range of strategies to maintain efficient functioning under an array of environmental conditions (Cairney, 1999; Nannipieri *et al.*, 2003).

Establishing whether diversity is important for ecosystem processes has become a central issue in ecology (Leake, 2001). In general, soil microbial communities appear to comprise groups of organisms that fulfill broadly similar ecosystem functions (i.e. exhibit functional redundancy) (Yin *et al.*, 2000). Functional diversity represents the value and range of capabilities that are possessed by organisms present in a given ecosystem and are relevant to ecosystem processes (Allen *et al.*, 2003; Sobek and Zak, 2003). There is a growing body of evidence suggesting that the functional characteristics of component taxa are at least as important as species richness for maintaining essential ecosystem processes (Naeem, 2002; Nannipieri *et al.*, 2003). Knowledge of the individual roles of mycorrhizal fungal species, or of their distribution either in relation to each other or to the physical and chemical environments of the soil, is limited (Goodman and Trofymow, 1998; Rosling *et al.*, 2003) and insufficient for determination of community needs and responses by building up from the species level. Moreover, in mycorrhizal ecosystems, we hypothesize that the functional significance of individual taxa is overshadowed by the integrated functional capability of the community, which is likely not an additive function of the independent capabilities of component species. The tendency to generalize ecological functions from a few fungal isolates reveals little information about the intrinsic physiological potential of most taxa (Cairney, 1999; Cairney and Meharg, 2003) or of the community. Current evidence suggests that ongoing parallel evolution of plant and fungal partners in response to environmental change on local and global scales may most readily explain extant patterns of mycorrhizal diversity and specificity (Cairney, 2000). Although functional redundancy almost certainly exists within mycorrhizal communities, high taxonomic and genetic diversity of ECM (and

probably ERM) fungi may indicate that they also exhibit a high level of functional heterogeneity (Cairney, 1999).

Are all the pieces of an ecosystem essential for restoration? Following a disturbance, should the management target be to maintain (or reintroduce) the original species richness at all costs, or, alternatively, to nurture the survivors (stress-resistors) so that they can contribute to the restoration of habitats in a future (altered) state? Is it likely that resistant organisms will modify the environment in ways that favour only themselves (i.e. preserving a specialized community), or do modifications lead eventually to succession by organisms that are incapable of tolerating the initial conditions (as suggested by most concepts of ecological succession)? Species richness may be a critical aspect of ecosystem resilience and functioning, but within a restoration context, more emphasis should perhaps be devoted to the resistant biota and their contribution in restoring pre-contamination conditions. Community specialization may indicate environmental stress, but we hypothesize specialization may also be a desirable response to stress, and a useful characteristic in allowing stressed ecosystems to achieve long-term stability and diversity.

Ecology of ecto- and ericoid mycorrhizal systems

Soil habitat

Soils are living, open, dynamic systems. They contain structured and heterogeneous matrices, generally store nutrients and energy, and support high microbial diversity and biomass (Nannipieri *et al.*, 2003). To thrive, soil microorganisms must mobilize energy and nutrients stored in soil. Soil structure provides a complex and variable set of microbial

habitats ranging from energy-rich to barren, or aerobic to anaerobic, over micrometre distances. Soil structure is determined by soil aggregation, which occurs when soil particles within aggregates cohere more strongly to each other than to adjacent aggregates (Hartel, 1998). Aggregates are composed of sand, silt and clay particles that are held together by organic matter, precipitated inorganic materials, microorganisms and the products of their metabolic activities (Griffiths and Caldwell, 1992; Hartel, 1998). Aggregates are dynamic, constantly forming and disintegrating. Organic substrates and plant residues are entrained and protected during aggregate formation and released during aggregate disintegration (Plante and McGill, 2002). The solid phase adsorbs important biological molecules (e.g. DNA, enzymes, etc.) and many soil reactions are catalyzed at the surfaces of soil minerals such as clays, Mn (III and IV) oxides and Fe (III) oxides (Nannipieri *et al.*, 2003). In addition, the zeta potential of charged mineral and organic surfaces generates a steep pH gradient around them. For example, McLaren and Skujins (1968) cite examples of the pH optima of enzymes being several units higher in colloidal systems than in solution, apparently due to the lower pH in the immediate environment of the enzyme, close to colloidal surfaces. Water occupies the aggregate pore spaces and forms a meniscus around a central pocket of air, which provides an aerobic and aqueous habitat suitable for supporting bacterial communities (Wardle, 2002). Pore water also retards gas exchange, thereby creating anaerobic microsites. Pore water also participates in hydrolysis and mediates other soil reactions (Hartel, 1998).

Boreal forest soils are typically acidic with seasonal or intermittent availability of mineral nutrients (N and P) and high C:N ratios due to the surface accumulation of recalcitrant

organic matter resulting from incomplete oxidation of plant material (Prescott *et al.*, 2000; Allen *et al.*, 2003). This organic layer (mor humus) stores nutrients and also contributes to moisture retention and soil structure (Prescott *et al.*, 2000). The forest floor is the most metabolically active fraction of these soils and is heavily colonized by ECM and ERM root systems of trees and understorey vegetation (Lundström *et al.*, 2000). Wallander *et al.* (2001) estimated the extraradical mycelia biomass of ECMs to represent about 820 kg ha^{-1} in boreal forest soils. Fungal metabolic activities produce organic acids that percolate with rain water down through the soil profile and contribute to accelerated weathering of mineral soils (Griffiths and Caldwell, 1992; Heinonsalo *et al.*, 2004). Soluble complexes are formed between the organic acids and Fe and Al ions in the upper mineral soil, thereby fostering leaching of Fe and Al ions and creating a weathered, eluvial horizon (Lundström *et al.*, 2000). These complexes percolate further downward and precipitate, creating a characteristic rust-coloured illuvial B horizon overlying the parent material (Lundström *et al.*, 2000). These changes with depth in soil chemical and mineralogical properties create contrasting habitats for microorganisms. For example, Rosling *et al.* (2003) found that the species composition of the ECM community varied between organic and mineral horizons of boreal podzolic soils and that most taxa occurred in only one part of the soil profile.

Less than 5% of the soil volume is occupied by microorganisms, but these sites of increased biological activity are where the majority of soil reactions are mediated (Díaz, 2004). The availability and nutrient content of organic matter are key factors influencing microbial biomass and community composition (Tiquia *et al.*, 2002). Other major factors controlling the distribution and abundance of soil microbial communities include: (1) properties of the

soil environment (e.g. pH, O_2 supply and availability of water and nutrients such as N, P, Fe); (2) factors affecting dispersal (e.g. soil structure, micro-aggregate stability and routes of dispersal); and, (3) the controls of population turnover (e.g. nematode or protozoan grazing, controls on lytic enzymes, protective soil matrices) (Tiedje *et al.*, 1999). Introduction of PHCs alters all three of these fundamental characteristics. For example, O_2 supply is often reduced, water movement is restricted and soil fauna including nematodes and protozoa are temporarily lost from the contaminated ecosystem.

Microbial growth in soils is typically resource-limited (most often energy-limited) and increases rapidly in response to addition of reduced C to provide energy for the large chemo-organotrophic biomass (Nannipieri *et al.*, 2003; Morgan *et al.*, 2005). Actively growing roots leak or secrete (exude) soluble and insoluble organic compounds into the surrounding soil (rhizosphere) that provide most of the low molecular weight C available to microorganisms (Darrah, 1991; Garbaye, 1994). Rhizodeposition is concentrated at the root tips and at sites of lateral branch formation, which correspond to sites of greater microbial population density and community complexity compared to bulk soil (Linderman, 1988; Chanway, 1997; Sarand *et al.*, 2000; Söderberg *et al.*, 2004). Soluble forms of C (e.g. monosaccharides, amino acids and organic acids) are readily metabolized by microorganisms to CO_2 or converted to biomass; insoluble forms of C (e.g. mucilages, sloughed cortical cells and dead root hairs) are less readily metabolized (Darrah, 1991), but they may form new microbial habitats, which are eventually consumed. As with fungi, bacterial richness and functional redundancy are both high, at least at coarse scales. Using fatty acid methyl ester profiles (FAME analysis) and 16S rRNA gene sequences, Axelrood *et al.* (2002*a*, *b*)

described immense bacterial richness (isolates representing 42 known bacterial genera and clones spanning nine divisions, respectively) in surface organic matter and mineral soil samples from forests in the central interior of British Columbia. Culture collections were well represented by *Pseudomonas*, *Bacillus*, *Paenibacillus* and *Arthrobacter* species (Axelrood *et al.*, 2002*a*), whereas molecular clones were represented by *Bradyrhizobium*, *Rhizobium*, *Pseudomonas* and *Burkholderia* species (Axelrood *et al.*, 2002*b*). These genera are considered common soil inhabitants and important components of rhizosphere communities with respect to nutrient cycling and transformation of minerals and complex organic substrates (Axelrood *et al.*, 2002*b*).

It has not yet been fully appreciated that the establishment of mycorrhizal symbioses substantially alters the morphology and physiology of plant roots (e.g. alters permeability of root membranes), which also changes root exudation patterns as well as the types of C substrates exuded (Linderman, 1988; Ingham and Molina, 1991; Rygiewicz and Andersen, 1994). The extraradical mycelia generate increased volumes of mycorrhizosphere soil compared to noncolonized roots and not only support microbial growth through exudation of energy-rich substrates, but also provide surfaces for colonization and contribute to formation of soil structure (Griffiths and Caldwell, 1992). The presence of ECM mycelia alters bacterial community structure by stimulating proliferation of selected bacterial populations, among other mechanisms (Frey *et al.*, 1997; Heinonsalo *et al.*, 2000). Fluorescent pseudomonads isolated from the mycorrhizosphere of Douglas-fir (*Pseudotsuga menziesii* (Mirbel) Franco) appear preferentially to use trehalose, a carbohydrate derived from fungal metabolism (Frey *et al.*, 1997). Fluorescent pseudomonads and actinomycetes have been

observed around ECM roots of birch, closely associated with the mantle and in proximity to fungal exudates (Ingham and Molina, 1991). There is also some evidence that diverse microbial communities may be selectively present in association with certain ECM mycelia (Garbaye, 1994; Read and Perez-Moreno, 2003). For example, Olsson and Wallander (1998) found that structure of soil bacterial communities, assessed using phospholipid fatty acid (PLFA) analyses, depended both on ECM fungal species and soil type. Fungal mycelial (mat) communities are unique soil habitats that contribute to maintenance of high richness of bacterial and fungal taxa within ecosystems (Griffiths and Caldwell, 1992).

In summary, conditions within soil habitats vary by orders of magnitude over micrometre distances, in response to physical (structure and aggregates), chemical (pH, O_2, soluble substances) and biological (microorganisms, soil fauna, plant roots) variables. Soil habitats may also be substrates (e.g. plant residues). Perhaps because of this almost infinite variety of habitats at the microbial-size scale, it is difficult to find any soil sample that is missing major genera of the known microbiota of terrestrial ecosystems. Molecular techniques continue to show increasingly large ranges of genetic material within soils (e.g. Axelrood *et al.*, 2002*b*; Prosser, 2002), with most (more than 99%) of the bacterial genotypes represented currently not culturable (Pace, 2005). With greater sampling effort, the number of known bacterial divisions has expanded substantially in recent years (Pace, 2005). From a management perspective, the genetic potential to mediate virtually any biogeochemical reaction and the habitat needed to support it appears to exist in most soils, with only specialized capabilities potentially missing.

Community interactions

Mycorrhizosphere bacteria

As mycorrhizal fungi constitute the most significant rhizosphere communities, they have immense potential for interactions with other soil organisms such as bacteria, fungi, protozoa, nematodes, arthropods and mammals, as well as with each other (Fitter and Garbaye, 1994; Read and Perez-Moreno, 2003; Cairney, 2005). The primary factors that influence the composition of associated communities are the quality and quantity of C compounds present, competitive interactions between mycorrhizal fungi and free-living microorganisms for mineral nutrients, and the beneficial, detrimental or neutral impacts of secondary metabolites produced by symbiotic or free-living organisms (Siciliano and Germida, 1998; Cairney and Meharg, 2002). The outcomes of interactions between ECM, ERM and saprotrophic fungal mycelia may include mutual interference of growth (deadlock) or replacement of one taxon with another through competition (Cairney, 2005).

The interactions between ECM/ ERM fungi and the heterotrophic bacterial community are important for accessing mineral nutrients (Burke and Cairney, 1998). Observations that enhanced decomposition of organic compounds occurs in (mycor)rhizosphere soils have been attributed to the greater metabolic activities associated with higher densities of microorganisms (Heinonsalo *et al.*, 2000). Enriched bacterial communities, often arranged as biofilms (organised systems consisting of layers of biologically active cells), have been noted at the surfaces of the ECM fungal mantle and extraradical mycelia, which are the sites of nutrient mobilization, uptake and translocation (Sen, 2003). The exposure of microbial biofilms to organic polymers such as cellulose and proteins appears to drive degradative

secondary metabolism; this enables plant and microbial uptake of simple compounds (e.g. sugars, amino acids and mineral nutrients) that are released during the decomposition process (Sen, 2003).

From the germination of fungal propagules in soil to establishment of true symbiosis, mycorrhizal fungi experience a free-living stage during which they interact with bacteria (known as mycorrhizal helper bacteria, MHB) that appear to be beneficial to the colonization process *via* one or more of several proposed mechanisms (Garbaye, 1994). In axenic culture with nutrient limitation, MHB may act by direct trophic interactions (where bacteria provide C substrates or growth factors to the free-living fungi) or by metabolic detoxification of fungal metabolites (e.g. polyphenols, etc.) (Duponnois and Garbaye, 1990). Bacteria that are active at the time of mycorrhizal formation may facilitate recognition between the plant and mycorrhizal fungus, improve the receptivity of the root for fungal colonization, or stimulate fungal growth, thereby increasing encounters between roots and mycelia (Frey-Klett *et al.*, 1997). MHB also appear to colonize fungal hyphae and stimulate initial mycorrhizal formation through production of vitamins, amino acids, phytohormones and/ or cell wall hydrolytic enzymes, which may influence germination and growth rates of fungal structures, enhance root development and/or decrease susceptibility to infection (Martin *et al.*, 2000). Shishido *et al.* (1996) found that three strains of fluorescent pseudomonads enhanced spruce seedling growth through mechanisms unrelated to increased mycorrhizal colonization, but growth promotion of pine by two strains was facilitated by an interaction with mycorrhizas. Mycorrhizal root tips tended to support slightly higher populations of *Pseudomonas* spp. than non-mycorrhizal root tips and additional colonization sites or altered/ enhanced exudation in

the mycorrhizosphere were observed. Frey-Klett *et al.* (1997) found that high levels of bacterial inoculum (MHB *Pseudomonas fluorescens* BBc6) in the rhizosphere are not necessary for a helper effect to occur.

Another group of naturally occurring, free-living soil bacteria that colonize roots and enhance plant growth when added to seeds and roots are known as the plant growth promoting rhizobacteria (PGPR) (Chanway and Holl, 1991). PGPR activity has been reported in *Azospirillum*, *Bacillus*, *Clostridium*, *Hydrogenophaga*, *Serratia*, *Staphylococcus*, *Streptomyces*, and *Microbacterium* species (Chanway, 1997). Holl and Chanway (1992) found that growth of mycorrhizal pine was stimulated by inoculating the rhizosphere with *Bacillus polymyxa* strain L6, which appeared to be a function of the size of the bacterial population. Plant growth promotion was not attributed to increased symbiosis by the ECM fungus *Wilcoxina*, and was also unlikely to be due to N fixation as this *Bacillus* strain contributed to only 4% of seedling foliar N. Rather, stimulation of pine growth may have been a result of bacterial production of plant growth substances such as indoleacetic acid. Other researchers have suggested that PGPR may, at least in the short term, improve the C supply to mycorrhizas by providing an increased supply of N (fixed from the atmosphere) to the plant (Ingham and Molina, 1991; Martin *et al.*, 2000). Microorganisms may directly stimulate plant growth by providing nutrients (e.g. N, P, S) or growth factors (e.g. auxin, cytokinin, gibberellin), increasing root permeability or inducing plant systemic resistance to pathogens. Indirectly, microorganisms may influence other rhizosphere components that influence plant growth, such as increasing legume or alder root nodule number and size,

increasing colonization frequency of mycorrhizal fungi, or suppressing deleterious rhizobacteria (Chanway, 1997).

Plant linkages

Plant communities in northern forest ecosystems are linked below ground *via* the extensive extraradical mycelial network of mycorrhizal fungi (Dahlberg, 2001; Simard and Durall, 2004). Host-specific fungi form intraspecific plant linkages, whereas fungi with more general host requirements may form interspecific linkages that allow for nutrient and C transfer between different tree species. In a microcosm experiment, radiolabelled C transfer through the soil mycelial network has been demonstrated between Sitka spruce [*Picea sitchensis* (Bong.) Carr.] and pine species (*Pinus contorta* Dougl. ex Loud. and *P. sylvestris* L.) (Finlay and Read, 1986). In the field, Simard *et al.* (1997) demonstrated bidirectional C transfer between Douglas-fir and paper birch (*Betula papyrifera* Marsh.) *via* a common mycelial network, with a significant net gain by the shaded Douglas-fir. Mycorrhizal networks appear to have the capacity to mediate significant N transfer among interconnected plants (*Casuarina* and *Eucalyptus* pairs); N gradients (between N-rich donors and N-limited receivers) may drive unidirectional N transfer (He *et al.*, 2005). Similarities in the composition of ECM communities associated with various host species in bioassays and field surveys indicate the potential for linkages between varieties of plant species (Kranabetter *et al.*, 1999; Massicotte *et al.*, 1999).

The coexistence of ECM and ERM plants in boreal forests provides many opportunities for sharing ECM and ERM fungi that link plants and translocate nutrients, although little

research on this issue has been conducted (Perotto *et al.*, 2002). Vrålstad *et al.* (2000) demonstrated that fungal strains derived from ECM morphotype *Piceirhiza bicolorata* constituted assemblages of very close relatives to ERM type *Rhizoscyphus ericae*. Similarly, Monreal *et al.* (1999) showed sequence similarity (ITS2 region) between the ECM fungus *Phialophora finlandia* and *R. ericae*. In a resynthesis experiment using 12 *R. ericae* strains on ECM and ERM hosts, Vrålstad *et al.* (2002*b*) showed that genetically close relatives of the ERM fungus *R. ericae* are true ECM partners with conifer (spruce and pine) and angiosperm (birch) species, but no isolates tested formed both ECMs and ERMs. These studies indicate that ECM and ERM plants may share mycobionts of this species complex (known as the *R. ericae* aggregate) and, based on ITS phylogeny, the ability to form both ECM and ERM symbioses may have evolved with the *R. ericae* aggregate. Villarreal-Ruiz *et al.* (2004) recently reported the ability of a fungus from the *R. ericae* aggregate to form simultaneously both ECMs and ERMs in culture with *Pinus sylvestris* and *Vaccinium myrtillus* seedlings, respectively, based on rDNA sequencing and microscopy.

Due to the complexity of the molecular mechanisms involved in establishment of a tight (host-specific) symbiosis, the type of fungal associations with different plant hosts may not be of great physiological importance under non-contaminated conditions, but may gain ecological importance under stressed environmental conditions (Perotto *et al.*, 2002). Mycelial linkages may influence fungal and plant ecology by providing a source of fungal inoculum to newly growing roots, allowing the C demands of the mycelium to be met by more than one plant and facilitating the transfer of C and mineral nutrients between neighbouring trees (Jones *et al.*, 2003). It has been proposed that ECM and ERM fungi may

contribute to development of plant communities if the net transfer of C and nutrients is predominantly from a pioneer plant species to a late successional species, but a greater awareness of these processes is important for understanding the interactions between trees and understorey vegetation (Dahlberg, 2001). Kernaghan *et al.* (2003) demonstrated a positive correlation between ECM fungal richness and overstory host tree richness that was explained by resource heterogeneity in combination with the preference (specificity) of ECM fungi for certain plant hosts. Recently, DeBellis *et al.* (2006) showed that the distributions of ECM fungi in southern mixed-wood boreal forests are influenced by the relative proportions of host tree species. Conservation of stand diversity should therefore support diverse fungal communities. Whether such communities are essential for ecosystem recovery following PHC contamination is still not known. Regardless, minimizing overstory disruption increases the possibility of preserving the integrated below-ground mycelial network with its associated communities, and maximizes its potential to hasten site recovery.

Ecosystem processes

Biogeochemical cycling of nutrients and energy through ecosystems is driven by ordering (e.g. photosynthesis, growth, humus formation) and dissipative (e.g. respiration, senescence, decomposition) processes (Addiscott, 1995). Mycorrhizal systems form the functional interface between decomposition (release of carbon and nutrients from organic substrates) and primary production (formation of biomass) for both above-ground and below-ground communities. In the below-ground food web, chemo-organotrophic organisms (those that obtain energy and carbon from organic substrates) appear to be ultimately responsible for governing nutrient availability for plant productivity (Wardle, 2002; Wardle *et al.*, 2004). In

reconstructed soil profiles (mini-ecosystems), the microflora (bacteria and fungi) were found to exert a greater influence on nutrient mobilization and tree growth than either the fungus-feeding mesofauna or predator trophic groups (Setälä *et al.*, 2000). Setälä *et al.* (2000) reported that although species composition of the trophic groups was important for system functioning, species richness within functional groups had a negligible impact on primary production. Soil fauna (nematodes, protozoa, enchytraeids, microarthropods, earthworms, termites, etc.) that feed on the microflora are also important in stimulation of primary production (Wardle, 2002) by preventing nutrient sequestration within inactive microbial biomass.

Decomposition

Heterotrophic bacteria and fungi directly decompose complex carbohydrates (mainly cellulose and lignin) in plant detritus (Wardle, 2002). Cellulose is readily biodegradable as it consists of β(1-4) linkages of D-glucose that form flat, linear chains H-bonded together to create microfibril sheets (Evans and Hedger, 2001). By contrast, lignin is a three-dimensional aromatic polymer consisting of β(0-4) linkages of monomeric units of either cinnamyl alcohol: coumaryl alcohol (grasses), coniferyl alcohol (gymnosperms) or sinaptyl alcohol (angiosperms) that surround the microfibrils and provide rigidity to plant cell walls (Evans and Hedger, 2001). Due to its complex and uniquely heterogeneous structure (i.e. hydrophobicity and thermodynamic stability), lignin is highly resistant to degradation (i.e. recalcitrant) and inhibits decomposition by up to a few years by limiting access of microorganisms or enzymes to substrates (Prescott *et al.*, 2000; Steffen, 2003).

In the early stages of decomposition, soluble compounds and cellulose are rapidly metabolized under conditions where C is available and N is usually limiting (Prescott *et al.*, 2000). Decomposition rates slow over time due to changes in substrate compounds (increased lignin fraction) and succession of microorganisms able to compete for various substrates (Berg, 2000). In the later stages of decomposition, there is a net loss of lignin and N is mineralized from humus (Prescott *et al.*, 2000). Growth may become N-limited in habitats with high C:N ratio substrates, whereas in habitats with low C:N ratio (<30:1) substrates, decomposition of organic matter may result in C limitation (Tiquia *et al.*, 2002). With the loss of cellulose, relative lignin and N concentrations increase and the higher N concentration can slow the decomposition rate. Such slowing may be due to low molecular mass N-containing compounds reacting with lignin residues during humification (Prescott *et al.*, 2000), creating more recalcitrant aromatic compounds; or mineral N may repress synthesis of lignin-degrading enzymes in a wide range of soil organisms (Gallo *et al.*, 2004); or a combination of these (Magill and Aber, 1998). Higher initial N concentration (lower C:N ratio) leads to lower decomposition extent (i.e. lower mass loss) and more stabilized organic matter in forest soils (Berg, 2000). The process of humus formation (i.e. humification) is thought to involve microbial modifications of lignin and condensation of proteins or amino acids into humus precursors, which polymerize into structurally intricate humus molecules (Prescott *et al.*, 2000). Compared to the original plant material, humus is low in carbohydrates (e.g. cellulose and hemicellulose) and high in polyphenolics (e.g. lignin constituents) and immobilized N, which is sparingly available to plants (Prescott *et al.*, 2000).

Although it is generally accepted that mycorrhizas play important roles in decomposition and cycling of C, N and P in ecosystems, details of their functions in nutrient dynamics and regulation of nutrient and energy flows are continuing to be developed and refined (Martin, 2001; Allen *et al.*, 2003). The traditional view of the role of mycorrhizas in obtaining limiting nutrients from forest soils involves fungal exploration for nutrients [e.g. amino acids, ammonium (NH_4^+), nitrate (NO_3^-) and inorganic P] that are released during decomposition of plant organic matter by heterotrophic fungal and bacterial communities or that are bound to the soil matrix (e.g. insoluble forms of Al and Ca phosphates) (Martin *et al.*, 2000). However, molecular studies have revealed that some fungal species, previously regarded as decomposers of woody debris (saprotrophs), are both frequent and abundant components of ECM communities (Hibbett *et al.*, 2000; Kõljalg *et al.*, 2000). This, along with other conceptual advances in biocomplexity theory, have led to re-evaluation of how mycorrhizas function within ecosystems and how interactions between multiple species of plants, mycorrhizal fungi and soil saprotrophs regulate community composition and ecosystem processes (Allen *et al.*, 2003).

It has been hypothesized that the distribution of the different mycorrhizal categories is related to specialization for nutrient acquisition in particular environments (Read and Perez-Moreno, 2003). In higher latitude and higher elevation forest ecosystems, where seasonally low temperatures and dry conditions result in very slow rates of decomposition, natural selection may have favoured ECM and ERM symbioses with the capacity to mobilize nutrients from organic material and provide them to plants (Read, 1991; Perotto *et al.*, 2002; Read and Perez-Moreno, 2003). Fungal specialization for N acquisition and utilization may be

important for determining community structure, lending strong theoretical support to the idea that ECM diversity increases the effectiveness of nutrient acquisition from different spatial locations and different substrates in soil (Martin *et al.*, 2000; Leake, 2001).

Some ECM fungi appear to be directly involved in nutrient mobilization from organic compounds through production of a wide range of hydrolytic and oxidative enzymes such as polyphenol oxidases (e.g. laccase, catechol oxidase and tyrosinase) and endochitinases (Martin *et al.*, 2000; Burke and Cairney, 2002; Lindahl and Taylor, 2004). Most ECM fungi so far investigated have demonstrated limited phenol-degrading activities, but few have been studied. By contrast, ERM fungi (e.g. *R. ericae*) appear to have well-developed saprotrophic abilities and degrade most polymeric components (e.g. polysaccharides, lignin, protein, chitin and pectin) of plant and fungal cell walls (Martin *et al.*, 2000). It is widely accepted that enzymatic degradation of organic polymers, through production of an array of hydrolytic enzymes in the extraradical mycelia and translocation of nutrients to the root, is the major benefit of ERM symbioses to plants (Perotto *et al.*, 2002). ERM plants may enhance their exploitation of complex soil substrates by broadening their metabolic capabilities through an association with several fungi endowed with different functional enzymes (Martin *et al.*, 2000). This implies that simultaneous associations with a variety of symbiotic fungi may be an important strategy to broaden the range of functions in the colonization of different substrates (Perotto *et al.*, 2002). Whereas many ECM and ERM fungi appear to have the ability to access N and P directly from organic compounds, the extent to which they contribute directly (*via* enzymatic catabolism) or indirectly (by influencing soil microbial

community structure) to decomposition remains unclear (Cairney and Meharg, 2002; Heinonsalo *et al.*, 2004).

Primary production

Regardless of the mechanisms involved, symbioses with mycorrhizal fungi improve plant nutrient acquisition by facilitating access to organic sources, potentially increasing the uptake of nutrients via extensive growth of the mycelia and circumventing nutrient depletion zones in the soil (Buscot *et al.*, 2000). Nutrients are absorbed across fungal membranes and are either retained by the fungi for biosynthesis and growth, or transported distances of centimeters to meters to the plant roots, where translocation to the host enhances the photosynthetic machinery of the plant (Allen *et al.*, 2003). Plant photosynthetic rates depend on the concentrations of N (for enzymes), P (for ATP and ADP), Fe and Mg (for chlorophyll), internal CO_2 and water (to keep stomata open to fix CO_2) (Buscot *et al.*, 2000). The resulting nutrient sink (root cells) allows for nutrient absorption and translocation through fungal cells that occurs more quickly than diffusion to the roots through soil (Martin *et al.*, 2000). It is thought that, as long as N or P is limiting in the soil environment, plants will support their fungal partners through continued allocation of C (Allen *et al.*, 2003).

By absorbing, assimilating and translocating nutrients (e.g. nitrate, ammonium and amino acids such as glutamate, glutamine and alanine), fungi create a C sink in the mycorrhizal roots (Allen *et al.*, 2003). Heinonsalo *et al.* (2004) recently reported equivalent ^{14}C allocations to roots and ECMs in organic and mineral A and B horizons of a podzol, using a *Pinus sylvestris* mini-rhizotron system. The sink strength controls the rate of photosynthate

production and the sugar supply appears to regulate some fungal gene expression (Buscot *et al.*, 2000). Carbon metabolism provides fungal mycelia and plant cells with the energy, reducing power and biomass required for synthesis of various metabolites (e.g. amino acids) required for growth (Martin *et al.*, 2000). Martin *et al.* (2000) have suggested that differences in ECM morphological features (e.g. abundant emanating hyphae with increased metabolic activity *versus* few emanating hyphae with decreased activity) may reflect differences in the need for C between taxa. Mycorrhizal fungi acquire most or all C *via* host photosynthesis and translocation (average 10-20% of net photosynthetic yield), but may also obtain C through assimilation following biodegradation of organic polymers in the soil (Martin *et al.*, 2000; Allen *et al.*, 2003).

The ecological significance of using organic polymers as C sources, which may decrease the need for C from the host plant, is unknown (Martin *et al.*, 2000). A substantial amount of fungal C is allocated to the synthesis of recalcitrant compounds such as chitin (60% of the fungal cell wall) that can persist in the environment for years and increase soil aggregation, stability, C storage and water-holding capacity. It has been estimated that as much as 20% of N in boreal podzolic forest floors may be retained in chitin (β-1,4 linked N-acetylglucosamine units) present in dead and alive fungal mycelia (Lindahl and Taylor, 2004). The remaining C is respired (43-60%), accumulated as fungal storage sugars (e.g. mannitol and trehalose) or lipids, or deposited in the mycorrhizosphere as labile compounds (sugars, amino acids) that support the growth of bacterial communities (Allen *et al.*, 2003; Heinonsalo *et al.*, 2004). The release of microbial communities from C limitation provides the potential for them to play a major role in decomposition and nutrient mobilization (Read

and Perez-Moreno, 2003). The C cost for maintaining the external mycelium is unknown, but the extent to which C is allocated from roots to mycelial systems may be intrinsically linked to growth and nutrient-foraging activities of ECM fungi. Using digital autoradiographic techniques, Leake *et al.* (2001) showed that patterns of C allocation within ECM mycelia are highly dynamic and responsive to changes in niche (caused by spatial variability in resource quality or interactions with other organisms). It has been hypothesized that coexistence among fungi may be explained by the differential partitioning of C resources among fungal species (Allen *et al.*, 2003). The key role of mycorrhizas in C cycling (particularly in the positive feedback loop between plant growth, decomposition and leaf litter quality) may have important consequences for the C gains and losses of ecosystems and thus for the C budget at local, regional and global scales (Read, 1991; Cornelissen *et al.*, 2001; Allen *et al.*, 2003).

Summary

The influences of mycorrhizal fungi on plant populations and communities are not merely the sum of effects on the individuals within populations (Dahlberg, 2001; Koide and Dickie, 2002). Due to their long history and multiple evolutionary events, different plants and fungi bring independent characteristics to the symbiosis, resulting in extensive physiological variation among mycorrhizas (Allen *et al.*, 2003). For example, some fungal species, previously regarded as saprotrophs, are both frequent and abundant components of ECM communities (Hibbett *et al.*, 2000; Kõljalg *et al.*, 2000). The spatial scales within which individual mycelia operate as physically or physiologically integrated entities in nature are also not clear (Cairney, 2005). For example, all groups of ERM fungi reported globally have

been found associated with salal from a single site (Berch *et al.*, 2002). Whereas a distinct suite of functions can be assigned to a single mycorrhiza, the many genomic combinations (genetic diversity) of symbionts, environmental heterogeneity and the extensive connectedness of mycorrhizal root systems result in a complex suite of ways that mycorrhizas can function in ecosystems (Cairney, 1999; Allen *et al.*, 2003). For example, certain species of mycorrhizal fungi, rhizosphere organisms and plants may interact such that there is a net immobilization of nutrients, which results in slower rates of decomposition (Allen *et al.*, 2003). Other combinations of organisms (guilds) may not influence the equilibrium of either nutrient cycling or decomposition, or may interact to increase nutrient quality of litter and decomposition rates.

High species richness and abundance may represent ecological adaptation to local environmental heterogeneity and is thought to provide forests with a range of strategies to maintain efficient functioning under an array of environmental conditions (Cairney, 1999; Nannipieri *et al.*, 2003). From a management perspective, a key challenge is to discover if modifications of the environment send mycorrhizal ecosystems in predictable directions. Or, alternatively, is it the combination of fungal and plant species that directs the trajectory? Current evidence suggests a vast range of genetic potential in most mycorrhizal ecosystems ready to respond to changing environmental conditions. Consequently, the hypothesis that environment is predominant in determining the outcome warrants testing.

Petroleum hydrocarbon contamination of forest soils

Disturbance

Analysis of changes to the structure and taxonomic diversity of soil communities following disturbance can provide clues to genetic and functional diversity by revealing some features of the surviving organisms. Although rarely considered in this way, discrete PHC contamination events such as oil spills are disturbances that disrupt ecosystem, community or population structure and alter the physical environment and resource or substrate availability (White and Pickett, 1985). Forest soil contamination is usually from a point source (i.e. involving discrete, localized and often readily measurable discharge of chemicals) and often results in rapid surface contamination, sometimes with large quantities of PHCs. For economic and toxicological reasons, the fate and behaviour of organic pollutants in soils has been the subject of intense research, with special interest focused on those chemicals that can be taken up or transformed by living organisms (Alexander, 1999; Semple *et al.*, 2003). However, the capacity of soil microorganisms to biodegrade organic pollutants not only depends on whether they have the necessary metabolic pathways, but also on whether the chemicals inhibit the microorganisms or are biologically available. Short- and long-term changes to soil microbial communities may result if some populations are susceptible to chemical toxicity (i.e. inhibition of cellular metabolic processes) or to changes to their physical (soil) and chemical (substrates/ inhibitors) habitat. Whether these changes alter community functions depends on the degree of redundancy within soil communities.

Chemical toxicity

Petroleum products are complex mixtures that can contain numerous aliphatic (linear and branched chains), alicyclic (unsubstituted and alkyl substituted structures) and aromatic (unsubstituted and alkyl substituted structures with at least one unsaturated ring) compounds (Miller and Herman, 1997; Potter and Simmons, 1998). Numerous methods are available for analysing petroleum hydrocarbon mixtures in environmental media (Weisman, 1998). Natural gases are generally composed of methane, ethane and small amounts of higher molecular mass hydrocarbons whereas most crude oils contain compounds such as paraffins, aromatics, naphthenics and asphaltenes that are present in varying proportions (McGill *et al.*, 1981; Potter and Simmons, 1998). Gasoline typically contains compounds in the nC4 to nC12 range, while diesel compounds are in the nC8 to nC21 range (Potter and Simmons, 1998). Light paraffinic crude oils are dominated by molecules with C numbers less than 16 and consisting mainly of alkanes (paraffins) and cycloalkanes (naphthenes). These chemicals are generally first metabolized by microorganisms (Westlake *et al.*, 1973; Riser-Roberts, 1998). The heavier oils have greater proportions of aromatic hydrocarbons and heterocyclic NSO compounds (i.e. containing N, S, or O) with C numbers usually above 20 (McGill *et al.*, 1981; Delille *et al.*, 2004).

Using chromatographic techniques, crude oils can be resolved into four categories of compounds: asphaltenes, saturates, aromatics and polars (eg. NSO compounds) (McGill *et al.*, 1981; Pollard *et al.*, 1992; Weisman, 1998). Asphaltenes are a mixture of pentane-insoluble, colloidal compounds including polyaromatic and alicyclic molecules with some alkyl substitutes (usually methyl groups) that vary in molecular mass between 500 and

several thousand (McGill *et al.*, 1981). The structures of these compounds, particularly those with higher mass, share characteristics with proposed structures of humic acids (Prescott *et al.*, 2000). Saturated and aromatic hydrocarbons (mainly n-alkanes, branched alkanes, mono-, bi-, and polycyclic alkanes (naphthalenes) and mono-, bi- and polyaromatics) usually account for 75% of the mass of crude oils (McGill *et al.*, 1981; Potter and Simmons, 1998). Up to 25% of the total mass may be n-alkanes, with cyclic hydrocarbons accounting for 30-60% of the total mass. Monocyclic aromatic compounds (e.g. toluene, benzene and xylene) and bicyclic types (e.g. naphthalene, biphenol) represent 1-2%; polycyclic aromatics (usually methylated derivatives of fluorene, phenanthrene, anthracene, chrysene, benzofluorene and pyrene) are present in lower amounts (McGill *et al.*, 1981). The NSO fraction contains polar compounds such as naphthenic acids, mercaptans, thiophenes and pyridines. Most of the N in crude oil is contained in the distillate residue as part of the asphalt and resin fraction and usually accounts for less than 0.2% (rarely exceeds 1%) by mass. The S content varies between 0.3 and 3% whereas the O content usually does not exceed 3% (McGill *et al.*, 1981).

Aliphatic compounds are generally less toxic than aromatics, and toxicity has been found to vary with compound size (McGill *et al.*, 1981; Edwards *et al.*, 1998). The quantity and composition of polycyclic aromatic hydrocarbons (PAHs) are major considerations in the evaluation of toxicity of PHC mixtures (Miller and Herman, 1997). PAHs with four or more benzene rings are known to be genotoxic to humans and other ecological receptors; in general, as relative molecular mass and polarity (i.e. degree of oxidation) of PAHs increase, carcinogenicity also increases and acute toxicity decreases. This is due to the metabolic production of highly reactive electrophilic intermediates that can access biological molecules

such as DNA, RNA and proteins and react to form adducts or lesions (Landis and Yu, 1995). Little is known about PHC toxicity to plant and microbial communities in forest soils as the majority of studies have excluded the complex interactions between combinations of chemicals, interacting communities and the soil environment that may exert synergistic, potentiative or antagonistic effects (Landis and Yu, 1995; Evans and Hedger, 2001; Koivula *et al.*, 2004). It is likely that toxicity varies with the type of pollutant, the extent of pollution and the general condition (i.e. extent of obvious signs of stress or disease) of the ecosystem prior to chemical disturbance (Seghers *et al.*, 2003).

In agricultural clay soils, the maximum toxicity of crude oil (indicated by worm survival, seed germination, bacterial bioluminescence and photosynthesis inhibition) was highest immediately after introduction of oil (Chaîneau *et al.*, 2003). An initial decrease in microbial density has often been observed immediately following the addition of PHCs to soil. For example, the addition of 10% (volume/ mass) toluene to soil resulted in survival of only about 1% of the indigenous bacteria, which eventually recolonized the soil to reach a high cell density (Huertas *et al.*, 2000). Biotransformations of PHC substrates may also lead to the release of an array of potentially toxic metabolites into the surrounding environment (McGill *et al.*, 1981; Riser-Roberts, 1998). For bacteria, toxicity resulting from PHC contamination has been inferred from decreases in enzyme (hydrogenase and invertase) activity (Suleimanov *et al.*, 2005), although reduced enzyme activities may also result from competition for limiting nutrients following PHC contamination.

The toxicity of non-ionized organic contaminants for microorganisms is primarily due to a nonspecific mode of action that involves partitioning of organic chemicals into the hydrophobic (lipophilic) layer of the cell membrane and disruption of membrane integrity (i.e. increased membrane permeability) (Miller and Herman, 1997). Kirk *et al.* (2005) suggested that interference with fungal membranes may explain the reduced extraradical hyphal growth of AM fungi (*Glomus* species) in PHC medium with soil. In general, fungi are considered to be more tolerant of high concentrations of polluting chemicals than bacteria, possibly due to differences in cell wall structure (Blakely *et al.*, 2002). Some yeasts (e.g. *Saccharomyces cerevisiae*) have been found to alter their membranes (i.e. increase hydrophilicity) to exclude hydrophobic contaminants (Park *et al.*, 1988). It is possible that similar compensation mechanisms occur in other fungi as well. Long-term resistance to PHCs could also be due to the ability of ECM fungi to produce spores that resist environmental stress factors and germinate when the concentration of toxicants associated with PHC contamination has decreased sufficiently over time (Nicolotti and Egli, 1998).

PHCs may directly kill plants on contact, slow their growth, inhibit seed germination, create nutrient-deficient conditions or, at lower concentrations, stimulate plant growth (McGill *et al.*, 1981). Nicolotti and Egli (1998) have suggested that crude oil has a caustic or lethal effect on plants only when it comes into direct contact with tissues and that reduced growth and biomass may be manifestations of changes to soil communities. In general, the taller the trees and the deeper their roots, the greater their tolerance to increased PHC concentrations in soil (Trofimov and Rozanova, 2003).

Soil properties and processes

Blakely *et al.* (2002) found that creosote impacted soil food webs and decomposition processes more by altering the habitat of microinvertebrates and their prey (i.e. fungi and bacteria) than *via* direct chemical toxicity. Kirk *et al.* (2005) suggested that PHCs may interfere with plant-fungus communication by altering root exudation patterns or changing the soil environment such that migration of diffusible chemical signals (e.g. flavonoids, auxins, etc.) is prevented. Many of the major impacts of PHCs on soil biota and plants in forest ecosystems appear to be associated with disturbances to water, nutrient and oxygen supplies related to the hydrophobicity and fluidity of oily products (Tarradellas and Bitton, 1997; Trofimov and Rozanova, 2003).

The disturbance caused by PHC contamination leads to considerable changes in physical and chemical properties that are not typical of unpolluted soils. PHC constituents may be found in mobile form, fixed in the soil pores and fissures, adsorbed on the surface of organic and mineral soil constituents or forming a continuous cover on the soil surface (Trofimov and Rozanova, 2003). Morphological changes in PHC-contaminated podzolic soils exhibit fragmentary patterns resulting from the unevenness of chemical distribution in the soil mass, increased amounts of iron in the upper horizons, and increased amounts of cemented soil aggregates (Trofimov and Rozanova, 2003). The extent of physical movement in the soil profile depends on temperature, PHC viscosity, moisture content, soil structure and soil texture (McGill *et al.*, 1981). Greater lateral spread of PHCs occurs in cold conditions; in hot and dry soil conditions, vertical movement into the water-unsaturated zone may occur more frequently (McGill *et al.*, 1981). In sandy soils, frontal migration of PHCs down the soil

profile along the paths of roots and fissures altered the soil profile to a depth of greater than 1 m (Trofimov and Rozanova, 2003). In gray forest soils, heavy fractions of PHCs were retained in the upper plow layer and filled the largest infiltration, aeration and drainage pores; lighter fractions were largely retained in illuvial horizons and filled fine water retention pores (Suleimanov *et al.*, 2005). This can lead to waterlogging and reducing conditions in the soil profile, both of which inhibit decomposition processes (Trofimov and Rozanova, 2003). Large amounts of oily material in soils may also indirectly increase soil temperature (by 1-10°C) if there is loss of surface vegetation. In some cases, more damage to the soil may occur due to the high osmotic potential of associated brine water (salinity 40,000 -45,000 $\mu g\ mL^{-1}$) than to the presence of PHCs alone (McGill *et al.*, 1981).

Water insolubility, hydrophobicity and soil sorptive properties increase with increasing size (number of aromatic rings) and complexity (molecule topology or pattern of ring linkages) of chemicals; PAHs with three or more rings tend to be strongly sorbed to the soil (Reilley *et al.*, 1996; Alexander, 2000; Kanaly and Harayama, 2000; Cerniglia and Sutherland, 2001; Chaîneau *et al.*, 2003). Chemical persistence in soil also depends on several environmental factors, including the type and quality of clay particles (as well as cation exchange capacity), the type and concentration of solutes in surrounding solution, soil organic matter (SOM) content and composition, pH and temperature (Alexander, 1999; Semple *et al.*, 2003). Organic chemicals may be sorbed to and retained by soil particles by adsorption or partioning. Adsorption entails chemical processes (ion exchange) or, more often, physical forces (H bonding or van der Waals forces) to surfaces of organic polymers or the external surfaces of 1:1 clay minerals and the external and internal surfaces of 2:1 (expanding) clays

(Alexander, 1999; Miller and Herman, 1997; Ellerbrock *et al.*, 2005). Sorption to minerals must compete with water and may be very low for nonionized organics in hydrated systems. Sorption to organic solids may occur *via* physical binding, which concentrates chemicals on outer surfaces or within the pores of a solid. Partitioning of organic chemicals into the SOM is a process of transfer from the bulk state of one phase to the bulk state of another by mechanisms analogous to dissolution and leads to a distribution of molecules within a portion or the entire volume of the organic matter (Alexander, 1999; Chaîneau *et al.*, 2003). The extent of chemical retention in the SOM fraction is directly correlated with the octanol-water partition coefficient of the substance (K_{ow}, measure of chemical hydrophobicity), the amount of SOM in the solid phase and its degree of oxidation or polarity (Xing *et al.*, 1994; Alexander, 1999; Wang *et al.*, 2005). In forest soils, the sequestration of organic pollutants in SOM (i.e. sorbed inside soil aggregates or at inactive particle surfaces) may decrease toxicity of chemicals through physical separation from biological receptors, which also decreases substrate bioavailability for enzymatic degradation (Alexander, 2000; Ellerbrock *et al.*, 2005). Toxicity of hydrophobic organic contaminants has been found to be less severe for organisms in soils with high humus content (Salminen and Haimi, 1997).

PHC-polluted soils are characterized by lower values of hygroscopic moisture, hydraulic conductivity and water retention capacity (i.e. wettability) compared to unpolluted soils (Trofimov and Rozanova, 2003; Suleimanov *et al.*, 2005). This is related to the spatial arrangement of hydrophobic components within SOM (Roy and McGill, 2000). Higher molecular mass components and their degradation products remain near the soil surface and form crusts that decrease water availability and limit water and gas exchanges between the

soil and the atmosphere. The creation of discrete and continuous water-repellent fronts parallel to the soil surface is also recorded in post-fire forest soils (Certini, 2005). Hydrophobic films on the exterior surfaces of soil aggregates reduce the wettability of the soil and increase structure stability (McGill *et al.*, 1981; Certini, 2005). Many PHC-contaminated soils eventually take up water and remain wet; however, long-term (years) hydrophobicity of crude oil contaminated agricultural soils has been documented in western Canada (Roy *et al.*, 1999).

The longer some chemicals remain in soil, the more they appear to resist desorption and biodegradation. Weathered (aged) chemical residues have considerable time to interact with the physical and chemical components of soil. Interactions may entail: (1) sorption, most likely *via* partitioning; or (2) irreversible incorporation into soil organic matter (*via* humification) by the catalytic activity of a variety of oxidative enzymes present in the soil matrix (Miller and Herman, 1997; Alexander, 1999). PHC pollution has been found to substantially increase the organic C (humic acid) content of soils (Trofimov and Rozanova, 2003). Humification of PHC constituents is explicit to transformation processes. Covalent bonding between organic chemicals and humic polymers (humin, fulvic acid and humic acid) in soil can form stable linkages to dialkylphthalates, alkanes and fatty acids that are resistant to microbial degradation and are not readily extractable with many organic solvents (McGill *et al.*, 1981; Alexander, 1999). Petroleum residues (as indicated by dichloromethane extraction) are associated with soil organic matter (Roy *et al.*, 2003). Sorption of volatile PHCs from adjacent soil has generated hydrophobicity in soils not directly contaminated with PHCs (Roy and McGill, 2000). They may be either directly incorporated through H bonding

of phenolic and benzene carboxylic acids into the molecular structure of soil humic materials or adsorbed to the surface of the molecule (McGill *et al.*, 1981). They are not entirely associated with the humic component, however, because exhaustive extraction with NaOH did not eliminate hydrophobicity of soils (Roy and McGill, 2000). It is not known whether complexes between hazardous organic chemicals and soil humic materials are cleaved in nature to give detectable levels of the original compound, whether these complexes are assimilated by animals and plants, or whether they pose problems of present or future toxicological significance (Alexander, 1999).

In PHC-contaminated soils, the C (energy) supply increases, which promotes metabolic activity on the part of all the microorganisms not directly inhibited by the PHCs and concurrently the C:N ratio tends to increase. The carrying capacity of a soil is the maximum level of microbial activity that can be supported under existing environmental conditions, which depends on the size of the population, availability of O_2 or nutrients, temperature and water availability. The carrying capacity of soils may be exceeded as a result of large inputs of C from PHCs (Miller and Herman, 1997). The intensive growth of PHC-oxidizing microorganisms in response to increased C availability is accompanied by consumption of soil nutrients, resulting in decreased nutrient availability for plants (Xu and Johnson, 1997; Tiquia *et al.*, 2002; Trofimov and Rozanova, 2003). A decrease in available N may also be partly due to the inhibition of nitrification and ammonification processes or from loss of nitrates (McGill *et al.*, 1981; Suleimanov *et al.*, 2005). Some studies have shown that nitrifying bacteria were not found in freshly contaminated soils, but that nitrification processes were eventually regained; stable organic matter and total N content have increased

significantly following PHC contamination (McGill *et al.*, 1981). An increase in total N has been attributed to increased atmospheric N_2 fixation during PHC biodegradation (McGill *et al.*, 1981). Thus, as the immobilization of mineral N present in the soil increases, the amount of available N decreases such that organisms benefiting from N_2 fixation, or consortia capable of recycling N from microbial biomass, are the only organisms that can thrive under these conditions (i.e. selection of PHC-tolerant species) (Nicolotti and Egli, 1998). Oxidative degradation may also alter the composition of soil bacterial communities, so that aerobic cellulolytic and proteolytic species decrease and anaerobic N- fixing species increase (Nicolotti and Egli, 1998). Under disturbed water and extreme O_2 limitation, P may be reduced and escape to the atmosphere as hydrogen phosphide (Suleimanov *et al.*, 2005), although this is a very small loss mechanism.

It does not appear that a single addition of PHCs limits microbial communities in the long term. However, several studies indicate that although total microbial numbers tend to increase over time, species richness often decreases, which may or may not have deleterious impacts on ecosystem functions (McGill *et al.*, 1981; Hofman *et al.*, 2004). In general, PHC contamination is expected to lead to an initial loss of richness, followed by rapid proliferation of metabolically competent members of communities inhabiting the new environmental conditions imposed by the chemical contaminants (Gramss *et al.*, 1998; Seghers *et al.*, 2003; Díaz, 2004). Some studies have reported drastic reductions in overall ECM biomass and colonization potential in soil following a spill, whereas some fungi appeared resistant to the PHCs and may have benefited from their presence (Nicolotti and Egli, 1998). In greenhouse experiments, spruce seedlings grown in crude-oil-contaminated soils exhibited shifts in ECM

community structure in response to increased contaminant concentrations (Nicolotti and Egli, 1998). Nitrogen availability may be a major factor structuring ECM fungal communities; mineral N and foliar nutrient ratios (N:P, P:Al) were found to be excellent predictors of fungal taxonomic richness in organic horizons and organic nitrate availability was a good predictor of their relative abundance (Lilleskov *et al.*, 2002). From studies of impacts of acid rain on soil communities, changes to soil chemical status and functions of the decomposer community have been suggested to lead to imbalances in nutrient cycling and ecosystem productivity (Pennanen *et al.*, 1998).

Biodegradation

The ability of heterotrophic bacteria and fungi to degrade organic pollutants appears to be inherent in most natural microbial communities and it is generally accepted that biological processes eventually degrade or transform most bioavailable (i.e. accessible by organisms or their enzyme systems) organic compounds (McGill *et al.*, 1981; Sarand *et al.*, 2000; Nannipieri *et al.*, 2003; Delille *et al.*, 2004; Díaz, 2004). Many xenobiotic chemical constituents (e.g. PHCs) are structurally analogous to compounds naturally found in the soil environment (e.g. plant material, fungal and root exudates and allelopathic chemicals) and appear to be biodegraded through the same biochemical pathways (Miller and Herman, 1997; Siciliano and Germida, 1998). In addition to accidental releases, low levels of PHCs may also enter soils from natural seepages or *via* atmospheric deposition after burning of fossil fuels (Knox *et al.*, 1999; Kanaly and Harayama, 2000; Trofimov and Rozanova, 2003; Certini, 2005). Biodegradative potential does not appear to be a distinguishing taxonomic character as metabolic ability (*via* different genes and biochemical pathways) is widespread

among many species of ubiquitous genera of bacteria and fungi (Siciliano *et al.*, 2003; Chaillan *et al.*, 2004).

The complete catabolic conversion (mineralization) of organic substrates to inorganic products (H_2O and CO_2) and use of nutrient constituents (C, N, P, S and other elements) for synthesis of cellular components is known as growth-linked biodegradation (Alexander, 1999). Biodegradation of some organic pollutants appears to result from transformations by microbial populations that are unable to use the substrate as a source of energy (even if the conversion is an energy-releasing oxidation reaction) or as an essential nutrient (i.e. not significant sources for biosynthesis) and is known as cometabolism (Miller and Herman, 1997; Alexander, 1999; Sarand *et al.*, 2000). Cometabolism is usually attributed to the activity of enzymes with relaxed specificities that act on structurally related substrates (Hickey, 1998). Organic substrates may be transformed to products that are not typical intermediates of central metabolism and the organism may not possess enzymes to convert further the compound into metabolic intermediates for biosynthesis or energy production. Alternatively, products may inhibit enzymes for subsequent metabolic conversions, suppress the growth of the organism, or the organism may require a second substrate (cofactor) to bring about a particular reaction (Alexander, 1999). Cometabolic reactions also have impacts in nature that are different from growth-linked biodegradation. Whereas the rate of growth-linked biodegradation characteristically increases with time as populations that are able to use the substrate as a source of energy and nutrients multiply, the environmental consequences of a population's inability to grow at the expense of the substrate are slow rates of biotransformation (due to small microbial biomass) and accumulation of organic products

that tend to persist in the environment (Alexander, 1999). The potential for substantial biodegradation of PHCs in soil (*via* growth-linked and cometabolic pathways) results in release of a nearly limitless array of metabolites into the soil environment. Some metabolites may be toxic to the soil biota; some may react with soil constituents or may be quickly degraded by other microorganisms present (McGill *et al.*, 1981; Riser-Roberts, 1998).

In a community context, biodegradation involves synergism. Syntrophic biodegradation occurs when two or more populations carry out transformations that one population alone cannot perform or performs slowly (Alexander, 1999). Thus, even if a particular population can metabolize only a small number of the chemical substrates available, other populations occupying the same habitat may possess complementary degradative enzyme capabilities that may ultimately result in complete chemical mineralization. Studies of mixed populations (i.e. communities) of bacteria have revealed more complex and rapid biodegradation than was previously believed possible based on studies of pure cultures (McGill *et al.*, 1981). Little is known concerning syntrophic biodegradation by specific guilds of organisms. If functional redundancy is the norm in most ecosystems, is the degradation of a substrate in soil dictated by specific guilds of organisms, or are the properties of the substrate and soil environment more important?

Bacterial pathways

Because of their metabolic versatility, bacteria are able to obtain energy from virtually every organic compound (Romantschuk *et al.*, 2000; Díaz, 2004). The most common electron acceptor for microbial respiration is O_2 and aerobic processes provide the highest amount of

energy to cells. Oxygen is not only the electron acceptor, but also participates in activation of the substrate *via* oxygenation reactions (Díaz, 2004). Anaerobic (anoxic) conditions are prevalent in aquifers, aquatic sediments and waterlogged soils. Here, biodegradation is carried out by strict anaerobes or facultative organisms using alternative electron acceptors such as nitrate (e.g. denitrifying organisms such as *Pseudomonas*, *Alcaligenes* and *Flavobacterium*), sulphate (e.g. sulphate reducers such as *Desulfobacterium*), Fe(III) (e.g. ferric iron reducers such as *Geobacter*), CO_2 (e.g. methanogens such as *Methanospirillum*) or others such as chlorate, Mn or Cr (McGill *et al.*, 1981; Díaz, 2004). Use of alternative acceptors depends on their availability as well as competition between different microorganisms for electron donors. The energy obtained using Fe(III) or nitrate is almost as efficient as using O_2, but less energy is generated by sulphate reducers and methanogens. Fermentative strains may be energy-limited and restricted to syntrophic existence, requiring other populations to consume the potentially toxic endproducts of fermentation. Photosynthetic organisms use energy from the sun to degrade aromatics anaerobically to acetyl-CoA, which is subsequently used in biosynthetic reactions (Díaz, 2004). In both aerobic and anaerobic degradation, structurally diverse compounds are degraded through many different peripheral pathways to a few intermediates that are further channelled *via* biochemical pathways (i.e. reactions leading to the formation of Krebs cycle intermediates) to the cell's central metabolism (Díaz, 2004).

Many microorganisms can use the aliphatic compounds present in PHCs as C sources. The mid-size straight-chain n-alkanes (nC10 to nC18) appear to be metabolized more readily than n-alkanes with shorter or longer chains, and saturated (single C bonds) are degraded more

readily than unsaturated (double C bonds) compounds (Miller and Herman, 1997; Delille *et al.*, 2004). The extent and location of hydrocarbon sidechain branching or halogen substitution slows the biodegradation of the compound (Miller and Herman, 1997). The most common aerobic biochemical pathway involves direct incorporation of one atom of O_2 into the alkane by a mixed function oxidase or monooxygenase enzyme, but both O_2 atoms can also be incorporated. In either case, a primary fatty acid is formed that is subjected to consecutive removal of two-carbon fragments (β-oxidation), which are converted to acetyl-CoA. This intermediate enters the Krebs cycle where complete mineralization to CO_2 and H_2O occurs (Miller and Herman, 1997). For alkenes, the first step is attack at the terminal or subterminal methyl group or at the double bond to yield an alcohol or epoxide that can be further oxidized to a primary fatty acid and enter β-oxidation. Degradation of alicyclic hydrocarbons (which are major components of crude oil, constituting 20-67% by volume) is thought to occur primarily *via* cometabolic reactions to open rings and subsequently cleave linearized products for entry into the Krebs cycle (Miller and Herman, 1997).

Under anaerobic conditions, however, oxygenation of hydrocarbons using O_2 is not possible. Aromatic ring structures may be activated under anaerobic conditions using a reductive rather than oxidative process. There is growing evidence that under anaerobic conditions some microbial communities are able to use O from H_2O or CO_2 for ring cleavage of aromatics or to prepare for ring cleavage of aromatics. For example, Schink *et al.* (1992) propose H_2O as the O source for ring cleavage during benzoate metabolism by fermenting and denitrifying bacteria, and CO_2 as the O source for carboxylation in preparation for ring cleavage of aniline by a sulphate-reducing bacterium.

Westlake *et al.* (1973) found that the ability of mixed populations of bacteria to use crude oil as a sole C source depended on the composition and amount of n-saturates, asphaltenes and NSO compounds and also that the aromatic fraction of crude oil was capable of supporting bacterial growth. It seems that PHC biodegradation is more closely related to the intrinsic biodegradability of chemicals (and their bioavailability) than to the particular enzymatic capacities of the microorganisms involved (Chaillan *et al.*, 2004).

Bacteria such as *Pseudomonas*, *Mycobacterium*, *Rhodococcus*, *Flavobacterium*, *Acinetobacter*, *Arthrobacter*, *Bacillus* and *Nocardia* are considered the primary degraders of polycyclic aromatic hydrocarbons (PAHs) in soil (Kanaly and Harayama, 2000; Chaillan *et al.*, 2004). *Pseudomonas* has been the most extensively studied, owing to its ability to degrade so many different contaminants and its ubiquity in soils containing PHCs (McGill *et al.*, 1981; Axelrood *et al.*, 2002*a*; Delille *et al.*, 2004; Díaz, 2004). Most aerobic peripheral pathways involve oxygenation reactions carried out by monooxygenases and/or hydroxylating dioxygenases that incorporate one or two atom(s) of O_2 into the aromatic ring structure to generate dihydroxy aromatic intermediates (e.g. catechol, protocatechuate, gentisate, hydroxyquinol and hydroquinone) (Miller and Herman, 1997; Siciliano and Germida, 1998; Cerniglia and Sutherland, 2001; Watanabe, 2002; Díaz, 2004). These compounds are the substrates of ring-cleavage enzymes that use molecular oxygen to open the aromatic ring between the two hydroxyl groups (*ortho* cleavage, catalyzed by intradiol dioxygenases) or proximal to one of the two hydroxyl groups (*meta* cleavage, catalyzed by extradiol dioxygenases). After several subsequent steps, the linearized products are

incorporated into the Krebs cycle. Anaerobic peripheral pathways usually converge to benzoyl-CoA, which is dearomatized by specific multicomponent reductases and consumes ATP (Díaz, 2004).

The initial hydroxylation step is considered to be rate limiting and the enzymes involved generally determine the substrate range of microorganisms, although other factors (e.g. substrate specificity of transcriptional regulators and membrane transporters) may also contribute (Watanabe, 2002). In a recent study of biodegradation by indigenous microbial communities in sub-antarctic soils, PAHs with greater than three rings were generally not degraded (Delille *et al.*, 2004). There is little evidence that microbial growth can be sustained with PAHs with four or more rings as a sole substrate (although they may be degraded by syntrophic cometabolism) and there is very limited information regarding bacterial degradation of PAHs with five or more rings (Reilley *et al.*, 1996; Kanaly and Harayama, 2000). However, bacterial degradation of pyrene (a pericondensed four-ring PAH) has been reported from sediment near a hydrocarbon source, and a *Mycobacterium* species isolated from that sediment has been shown to degrade pyrene using an inducible enzyme system (Kanaly and Harayama, 2000).

The metabolic flexibility of bacteria is related to their genetic adaptability. For example, Siciliano *et al.* (2003) found that a substantial decrease of aged PHCs in soil was related to a greater presence of catabolic genes (i.e. *alkB*, alkane monooxygenase; *ndoB*, naphthalenedioxygenase; *xylE*, catechol-2,3-dioxygenase) in bulk and rhizosphere soil. However, it was unclear whether the number of organisms containing these genes increased,

or if the number of genetic elements present in the community increased. The genes responsible for aromatic biodegradation pathways are usually arranged in clusters (operons) in mobile genetic elements (e.g. plasmids or transposons). Gene clusters contain catabolic genes (encode enzymes for catabolic pathways), transport genes (responsible for active uptake of the compound) and regulatory genes (adjust expression of the catabolic and transport genes to the presence of the compound to be degraded) (Díaz, 2004). For example, the catabolic genes of the *ortho* and *meta* pathways are organized as operons with flanking transposon elements on the TOL (toluene) plasmid (Sarand *et al.*, 1998). This facilitates horizontal transfer of the respective genes and rapid adaptation of microorganisms to the presence of new substrates (Díaz, 2004). Conjugation (transfer of genetic material from one microorganism to another) appears to be important in the dissemination of catabolic genes in the indigenous environment (Sarand *et al.*, 2000; Siciliano *et al.*, 2003).

Depending on chemical structure, contaminant concentration and environmental conditions, the onset of PHC biodegradation generally follows a period of acclimation in which no chemical degradation is evident (Alexander, 1999). Adaptation most commonly occurs by induction of the enzymes necessary for biodegradation, followed by increases in populations of biodegrading organisms (Miller and Herman, 1997). Chronic exposure to PHC substrates (e.g. near natural seepages or in areas where frequent spills occur) results in shorter acclimation periods (due to maintenance of biodegradation pathways within adapted communities) and subsequently increased transformation rates (Miller and Herman, 1997; Alexander, 1999). This pollution-induced community tolerance appears to increase proportionally with increased exposures (Seghers *et al.*, 2003). The end of this period is

marked with a rise in respiration and increase in density (varying from slight to several orders of magnitude) that reflects growth of hydrocarbon-degrading populations as well as increased growth of organisms such as protozoa that graze microflora or decompose necrotic tissue (McGill *et al.*, 1981). Subsequent declines in microbial respiration may occur due to complete degradation of labile fractions or to limiting availability of N and P (McGill *et al.*, 1981). High abundance, rapid growth and the ability to transfer genes horizontally allow for rapid microbial adaptation to changes in environmental conditions (Romantschuk *et al.*, 2000; Díaz, 2004).

Genetic changes such as mutations (i.e. appearance of new genotypes) may occur when communities are faced with chemicals that do not have natural chemical analogues (Miller and Herman, 1997). Such events occur at low frequency; however, if new genotypes possess physiological characteristics that provide a selective advantage (e.g. new metabolic capacities), they may multiply (*via* horizontal gene transfer) within the surviving community (Alexander, 1999). The length of time required for a genetic change or for selection and development of an adapted community is not yet predictable (Miller and Herman, 1997). However, given enough time and favourable environmental conditions, the capacity to degrade almost any organic compound is likely to evolve in or immigrate to a contaminated site (Romantschuk *et al.*, 2000).

Fungal cytochrome P450 and ligninolytic systems

Many fungi (e.g. *Aspergillus*, *Penicillium*, *Fusarium*) isolated from PHC-contaminated soils and cultured on PHC-containing medium have been found to use crude oil as a sole C and

energy source (Chaillan *et al.*, 2004). In general, eukaryotic organisms oxidize aromatic compounds to water-soluble products *via* a cytochrome P450 monooxygenase reaction, incorporating one atom of molecular O_2 into the aromatic ring to form a transient arene oxide and reducing the second atom of O_2 to H_2O. The arene oxide is immediately hydrated by an epoxide hydrolase to yield a trans-dihydrodiol or, alternatively, is non-enzymatically isomerized to form phenols that can conjugate with sulphate, glucuronic acid or glutathione (Miller and Herman, 1997; Cerniglia and Sutherland, 2001). These reactions increase both the water solubility and bioavailability of chemical substrates; soil conditions that favour fungal activity may initially increase the toxicity of the parent chemicals (Reilley *et al.*, 1996). Whereas complete mineralization results in innocuous endproducts (CO_2 and H_2O), partial biodegradation can produce intermediate metabolites with unchanged, reduced or increased chemical toxicity. Toxic chemical intermediates with increased water solubility are of particular concern as this can result in the transport and spread of contaminants through the environment (Miller and Herman, 1997).

The ligninolytic enzyme system of white rot fungi (WRF) has been extensively studied due to structural analogies between lignin and PAHs as metabolic substrates (Scheel *et al.*, 2000). Although lignin is a much larger and more heterogeneous polymer than the fused benzene ring structures of PAHs, it is also hydrophobic and insoluble, thereby posing similar problems for enzyme catalysis (Harvey and Thurston, 2001). WRF degrade lignin using a complex nonspecific enzyme system, often while simultaneously obtaining C from cellulose and hemicellulose (i.e. cometabolism) (Scheel *et al.*, 2000; Steffen, 2003). As with the cytochrome P450 system, the oxidizing enzymes of the ligninolytic system increase the

bioavailability, solubility and redox status of the chemical substrates for subsequent metabolism (Harvey and Thurston, 2001). Different fungi appear to possess different combinations of oxidizing enzymes (Harvey and Thurston, 2001).

The initial hydroxylation step of the pathway is accomplished with small, diffusible oxidizing agents (highly reactive radicals) generated by three groups of extracellular enzymes: lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases (Collins and Dobson, 1997; Harvey and Thurston, 2001). LiP (EC 1.11.1.14) and MnP (EC 1.11.1.13) are heme-containing enzymes that function at low pH and catalyze the oxidation of lignin, humic substances and many organopollutants (Schlosser and Höfer, 2002). Both enzymes require H_2O_2, which is generated through fungal glucose oxidase, glyoxal oxidase and arylalcohol oxidase reactions (LiP and MnP) or oxidation of organic acids (MnP only) (Evans and Hedger, 2001). In the white rot basidiomycete *Phanerochaete chrysosporium*, PAHs with ionization potentials at or below about 7.55eV are substrates for direct one-electron oxidation by LiP, whereas those with ionization potentials above this threshold appear to be acted upon by radical species formed during MnP-dependent lipid peroxidation reactions (Bogan, Schoenike and Lamar, 1996). For LiP, radical cations are produced from one-electron oxidations of non-phenolic compounds, which act as non-specific redox mediators and extend the substrate range and redox capacity of LiP (Harvey and Thurston, 2001). During the catalytic cycle of MnP, the active centre is oxidized by H_2O_2. Reduction of the resting enzyme is achieved by two successive one-electron transfers that oxidize Mn^{2+} to Mn^{3+}, which is facilitated by fungal organic acids (e.g. oxalate or malonate) upon chelation of the highly reactive Mn^{3+} state. Schlosser and Höfer (2002) found evidence supporting a

physiological role of laccase-catalyzed Mn^{2+} oxidation in providing H_2O_2 for extracellular oxidation reactions and demonstrated a novel type of laccase-MnP cooperation relevant to biodegradation of lignin and organic pollutants.

Laccases (benzendiol:oxygen oxidoreductase, EC 1.10.3.2) are (blue) multicopper enzymes (glycosylated polyphenoloxidases) that are an essential component of a complex nonspecific enzyme system secreted by different kinds of fungi that have been shown to oxidize lignin and various organic contaminants (Schlosser and Höfer, 2002; González *et al.*, 2003; Hoegger *et al.*, 2004). In addition to lignin depolymerization and polyphenol degradation, laccases are thought to be involved in the release of N from insoluble protein-tannin complexes, mycelial pigmentation, humus formation, fruiting body formation and detoxification of phenolic compounds, which protects fungi against soil pollutants and host defense compounds (Kanunfre and Zancan, 1998; Burke and Cairney, 2002; Hoegger *et al.*, 2004). Most reports refer to laccase activity as extracellular, but some WRF may also have intracellular laccases (Burke and Cairney, 2002). Laccases catalyze the reduction of O_2 to H_2O (4 electron reduction without formation of free reduced oxygen species) using a range of phenolic compounds as hydrogen donors (Burke and Cairney, 2002; Schlosser and Höfer, 2002). An electron is removed from the phenolic hydroxyl groups of lignin to form free phenoxy radicals, which are further oxidized to quinines (Hoegger *et al.*, 2004).

Metabolic potential of ECM/ ERM fungi

As some ECM and ERM fungi are closely related to WRF, some researchers have suggested that they may have retained some ability to degrade organic substrates, including PHCs

(Hibbett *et al.*, 2000; Meharg and Cairney, 2000; Meharg, 2001). Braun-Lüllemann, Huttermann and Majcherczyk (1999) reported on the ability of 16 species (27 strains) of ECM fungi that were isolated from middle European forests to degrade PAHs (1500 ppm of phenanthrene, pyrene, chrysene and benzo[*a*]pyrene) in pure, liquid culture. The slow but efficient metabolism of benzo[*a*]pyrene by ECM fungi was comparable to results of experiments with WRF (Braun-Lüllemann *et al.*, 1999). A study in which 58 fungal isolates from different physio-ecological groups were exposed to a range of PAHs showed that all fungal groups could degrade PAHs, but that ECM fungi were 19% as efficient as WRF (Gramss *et al.*, 1999). Similarly, Meharg and Cairney (2000) tested 42 ECM fungal species with several types of persistent organic pollutants and found that 33 species were able to degrade one or more classes of chemicals. Only one of 21 ECM fungal species could not degrade at least one PAH; degrading species seemed to prefer chemicals with four to five rings. In each case, the direct oxidative activities correlated with production of extracellular enzymes that appeared to metabolize aromatic rings (Braun-Lüllemann *et al.*, 1999; Gramss *et al.*, 1999). Green *et al.* (1999) reported that the ECM fungus *Tylospora fibrillosa* degraded 4-fluorobiphenyl to significant extents *via* sequential hydroxylation reactions.

A variety of ECM and ERM fungi are known to produce polyphenol oxidases (e.g. laccase, catechol oxidase and tyrosinase) in culture conditions, but there is little evidence for production of extracellular peroxidases (e.g. LiP and MnP) (Cairney and Burke, 1998; Burke and Cairney, 2002). Timonen and Sen (1998) assayed macerated fungal mycelia from several regions of a microcosm system and found that levels of enzyme activity in the environment were lower for ECM fungi than for saprotrophic fungi, possibly due to

avoidance of competition or preferential exploitation of substances of a particular quality. Gramss *et al.* (1998) reported that most cultured ECM fungi (from sporocarps collected from previously contaminated forest plots and industrial sites) exhibited oxidase activity: high extracellular enzyme activities were found for *Lactarius* and *Russula* species and high intracellular enzyme activities were found for *Suillus*, *Hebeloma*, *Leccinum* and *Tricholoma* species. Donnelly and Entry (1999) found extracellular enzyme activity at the advancing hyphal front of ECM fungi and suggested the possibility of a lack of complete dependence for C on the plant partners.

Few studies have considered mycorrhizal fungi in symbiosis with a plant for the degradation of organic pollutants (Koivula *et al.*, 2004). Meharg *et al.* (1997) found that the mineralization rate of ^{14}C-labelled 2,4-dichlorophenol by ECM fungi (*Suillus* and *Paxillus* species) in symbiotic culture with *P. sylvestris* seedlings was increased by 50% and 250% compared to respective rates of those ECM fungi in axenic culture. As mineralization was extremely slow in vermiculite (i.e. no bacteria present), these data were interpreted to suggest that fungal patch differentiation led to greater enrichment and stability of bacterial communities at the fungal-soil interface. It is unknown how the C contributions of the phytobiont influenced fungal responses or how synergistic or antagonistic interactions between mycorrhizas and other microorganisms altered their ability to mineralize or degrade organic pollutants.

Genetic controls

Ligninolytic enzymes are typically produced by WRF as multiple isoenzymes. This biochemical diversity had been attributed to post-transcriptional modifications of a single gene product, but characterization of several laccase gene families suggests that at least part of this diversity could be due to the multiplicity of laccase genes in fungal genomes (González *et al.*, 2003). For example, Hoegger *et al.* (2004) found that *Coprinopsis cinerea* has at least eight different laccase genes within the haploid genome, which is the largest laccase gene family reported so far from a single haploid fungus. Bogan *et al.* (1996) found that the genome of *Phanerochaete chrysosporium* contains at least 10 structurally related genes (*lipA* through *lipJ*) encoding LiP proteins and at least three MnP genes (*mnp1*, *mnp2*, *mnp3*).

Screening genomes for genes that encode laccases and peroxidases may represent a reliable means of identifying potential enzymatic activities in ECM and ERM fungi (Burke and Cairney, 2002). As DNA sequences for laccase and peroxidase genes are now available for several saprotrophic fungi, opportunities exist to design molecular probes or primers for identification of similar genes and/ or mRNA transcripts in mycorrhizal fungi (D'Souza *et al.*, 1996; Burke and Cairney, 2002). For example, Chen *et al.* (2003) used laccase gene primers to screen ECM basidiomycetes for laccase-like genes, which were amplified from *Lactarius*, *Russula*, *Piloderma* and *Tylospora* species. Timonen and Sen (1998) examined gene expression in identified functional components of *P. sylvestris* mycorrhizal systems and found expression of isozymes (i.e. polyphenol oxidase and acid phosphatase) was increased in hyphal fronts of *Paxillus involutus* and *Suillus bovinus* ECM systems as they advanced in

the humus. Through gene amplification and sequencing, Chambers *et al.* (1999) reported evidence for MnP genes and peroxidase activity in cultured *Tylospora fibrillosa*. Chen *et al.* (2001) extracted DNA from dried basidiomes or fungal cultures and amplified and sequenced genes for LiP and MnP using primers based on *Phanerochaete chrysosporium* genes. Although they reported the presence of LiP genes in a broad range of ECM fungal taxa and MnP genes in some ECM fungal taxa (three Atheliaceae taxa), Cairney, Taylor and Burke (2003) recently attempted to repeat these experiments and reported a lack of evidence to support the presence of peroxidase genes in ECM fungi.

Extracellular laccase is constitutively produced in small amounts by several fungi, but enzyme expression is considerably enhanced by a wide range of substances, including a variety of different aromatic compounds (Burke and Cairney, 2002; González *et al.*, 2003). Regulation of laccase production appears to be complex and vary between taxa. For example, in the WRF *Pycnoporus cinnabarinus*, laccase activity is increased with an increase in C:N ratio whereas in *Phanerochaete chrysosporium*, laccase activity is repressed by glucose regardless of N content and increased in the presence of cellulose when N is also increased (Burke and Cairney, 2002). From assays of liquid cultures of the ECM fungus *Thelephora terrestris*, Kanunfre and Zancan (1998) found increased secretion of extracellular laccase with a decreased C:N ratio. At the molecular level, Collins and Dobson (1997) demonstrated that laccase gene (*lcc*) transcription was activated by copper and nutrient N and that induction occurred at the level of gene transcription in the presence of two aromatic compounds. Chen *et al.* (2003) reported that some laccase-like genes amplified from

Lactarius, *Russula*, *Piloderma*, and *Tylospora* species appeared to be regulated at the transcriptional level, with transcription enhanced by higher N content.

Implications for management

Two broad objectives dominate management goals for contaminated forest sites: (1) reduction of risk; and, (2) long-term forest sustainability. In Canada, as in many jurisdictions, contaminated site remediation is required if there is risk to human or environmental health; this is not well defined for forest ecosystems and has led to situations of either over- or under-management. Here we wish to reflect on both these objectives and show how they converge.

Risk arises from the conjunction of three conditions: a contaminant (toxicant), a pathway and a receptor. Mycorrhizal ecosystems in contaminated sites may attend to the toxicant by metabolizing and removing or immobilizing it, or by transforming and altering its mobility and toxicity. Moreover, mycorrhizal ecosystems may also be among the critical receptors. Consequently, the concept of risk entails both remediation and ecotoxicology.

Bioremediation can be defined as the use of organisms to detoxify contaminants through immobilization, chemical transformation and mineralization processes (Díaz, 2004). Bioremediation efficacy is influenced by a variety of substrate and soil conditions, including: PHC composition; soil temperature, texture and structure; length of time the PHCs have been in the soil; and associated bioavailability, together with associated toxicants (Pollard *et al.*, 1994). Use of microorganisms as management tools requires knowledge of which organisms

(or functional guilds of organisms) are likely to be present in a particular ecosystem, how they respond to different types of physical and chemical disturbances, and methods for ascertaining whether organisms are actually healthy and not just surviving (Blakely *et al.*, 2002). From previous sections of this review, we can ask: (1) does the genetic potential exist (or is it likely to exist) to metabolize the array of substrates expected in the PHCs at a given site; (2) if so, what environmental conditions are likely to foster its expression and can these conditions be achieved; (3) is there need for added genetic potential through genetic engineering technologies; (4) if so, what constraints might limit its expression and what precautions might be needed; (5) is bioaugmentation needed to increase genetic potential; and, (6) if so, what precautions might be necessary, and how might its potential be best exploited? Although much remains unknown, considerable insights have been gained from recent and ongoing research regarding these questions.

Observations that virtually all organic substrates appear to be transformed by soil microorganisms if they are accessible (e.g. Simard *et al.*, 1997; Read and Perez-Moreno, 2003; Díaz, 2004; Heinonsalo *et al.*, 2004), combined with the continually increasing diversity of soil microbial communities revealed by molecular techniques (e.g. Axelrood *et al.*, 2002*b*; Berch *et al.*, 2002), the observation that catabolism of PHCs and plant residues share many common elements, and the absence of reports of soils that lack the ability to metabolize PHCs, all point to the ubiquitous genetic potential by soil communities to transform and perhaps completely catabolise PHCs. Consequently, bioremediation appears to be a sensible and potentially feasible intervention and has been extensively used. The focus of bioremediation strategies tends to be on contaminant disappearance, but is based on

a limited understanding of links between degradation and the basic nutritional needs of the responsible soil microbial community (Mills *et al.*, 2003). In addition, the optimum environmental conditions to sustain communities that degrade PHCs are understood only in broad terms. Continued progress may be expected by careful attention to, and documentation of, the connection between the environmental conditions imposed on a site by PHC contamination, as well as catabolic response and environmental preference by persistent communities. In essence, PHCs impose their own environment, including varying concentrations of toxicants. Consequently, PHCs control the community or guild that survives, which in turn dictates the optimum conditions for its functioning. Based on first principles, and observations on a wide range of chemo-organotrophic microorganisms, it is reasonable to expect that a slightly acidic pH, well oxygenated and nutrient sufficient environment would favour functional guilds that would metabolize PHCs. Attaining such conditions, however, can be challenging in forest ecosystems without disrupting them.

Is there a need to use recombinant organisms? In the early development of bioremediation technology, investigators recognized the scope of environmental pollution and the diversity of chemical pollutants, and invested significant effort into metabolic engineering to manipulate specific catabolic pathways or particular host cells (Alexander, 1999). Metabolic engineering has created recombinant organisms with novel hybrid pathways of biodegradation and increased substrate ranges; it has completed incomplete pathways, created multiple pathways, and provided mechanisms that enhance chemical bioavailability (Díaz, 2004). Because of issues (e.g. biosafety or inability to compete for resources) associated with introducing recombinant bacteria to contaminated ecosystems,

bioremediation (using recombinant bacteria) is often conducted *ex situ*, under relatively controlled conditions (Díaz, 2004). *Ex situ* bioremediation (with or without recombinant bacteria) is also used in situations where a high degree of control of environmental conditions is wanted (Riser-Roberts, 1998) and where added energy inputs such as in rotating bioreactors are desired. Recombinant organisms face public resistance due to the fear of their escape from the site, or transfer of genetic material to indigenous populations. Further, they may not always compete well with indigenous populations, or may require specialized environments. Consequently, they may be of limited potential for use on a large scale in forested ecosystems.

There are fewer biosafety issues associated with bioaugmentation, the introduction of exotic microorganisms isolated from unrelated sites, but adapted to contaminant biodegradation (Ward *et al.*, 2003) or to extreme soil conditions (Cunningham *et al.*, 2004; Stallwood *et al.*, 2005). Such additions can be made in a variety of ways, including industrial production and subsequent slurry applications. Soil samples from adjacent contaminated and remediated sites used as an inoculum would seem to be reasonable candidates as well. Although it can be readily used in the field, bioaugmentation lends itself better to highly engineered systems (e.g. slurry bio-reactors), to recalcitrant or novel contaminants for which the indigenous population may be ill-equipped, or for extreme environments (e.g. Cunningham *et al.*, 2004; Stallwood *et al.*, 2005). Cost is also a factor from a management perspective. Leavitt and Brown (1994) reported on three case studies comparing bioaugmentation using a commercial supplement with stimulation of indigenous soil organisms for removal of PHCs in a bioreactor and a land-treatment facility, and acetone or *bis*-2-chloroethyl ether in a waste-

water facility. Based on cost and efficacy, they concluded that bioaugmentation was not warranted in their situations and that biostimulation of indigenous organisms was the best choice. *In situ* bioremediation through enhancing contaminant biodegradation by indigenous soil populations and communities (i.e. syntrophic bioremediation) is considered less destructive and more cost-effective for remediating contaminated soils on large scales (Delille *et al.*, 2004; Doelman and Breedveld, 1999) and has proven successful when properly implemented (Nelson *et al.*, 1994). *In situ* bioremediation is more likely to maintain the desired integrity of below ground mycelial networks.

Phytoremediation refers to all plant-induced biological, chemical and physical processes that aid in the remediation of contaminants (Cunningham *et al.*, 1996). Traditionally, research in this area has focused on use of agricultural plant species for the remediation of agricultural or industrial soils. Reilley *et al.* (1996) found that the presence of vegetation significantly enhanced the dissipation (and likely biodegradation) of anthracene and pyrene in the soil environment. Others have reported that various grasses, legumes and woody plants facilitate the degradation of PHCs in soil (Aprill and Sims, 1990; Chaîneau *et al.*, 2000; Liste and Alexander, 2000; Palmroth *et al.*, 2002; Merkl *et al.*, 2005). Plants and associated mycorrhizospheres may increase the activity of PHC-degrading organisms, either *via* general enhancement [i.e. (mycor)rhizosphere effect] or due to proliferation of specific microbial groups (i.e. altered functional component of the microbial community) (Siciliano *et al.*, 2003). They may also mediate desorption of contaminants bound to soil constituents by altering pH and redox potential, as well as concentration and types of organic compounds in the (mycor)rhizosphere. Research has shown that sites containing plants and expected

mycorrhizal associations experience more rapid reduction in toxicity of PHCs (Parrish *et al.*, 2005).

An increased awareness of the abundance and diversity of mycorrhizal systems in vegetated soils has led to their consideration for *in situ* bioremediation (Meharg, 2001). Where there is little risk to human or ecological health, the purposeful planting of trees inoculated with specific mycorrhizal fungi is expected to establish these mycelial systems in soil and allow gradual decontamination over a period of several years (Braun-Lüllemann *et al.*, 1999; Meharg and Cairney, 2000). A related approach is to transplant plugs or sprigs of vegetation from non-contaminated soil, as is done in various restoration ecology projects (e.g. Fraser and Kindscher, 2005), into contaminated areas for the final stages of clean up. This approach allows for simultaneous remediation and revegetation of sites without further disruption to physical and chemical properties of the soil and provides an inoculum of a soil community adapted to the site. Although it may require several months or years for tree root systems and associated mycorrhizal biomass to establish, mycelial systems would be expected to remain in a vital state for several decades, whereas other organisms (e.g. WRF) may complete their life cycles in a few days or weeks and then rest as spores (Gramss *et al.*, 1999). A more thorough knowledge of which fungal symbionts are likely to survive and compete in various ecosystems, as well as which fungi contribute directly (exhibit biodegradative capabilities) or indirectly (provide suitable habitat for other microorganisms that exhibit biodegradative capabilities) to bioremediation of contaminated sites is required as part of management strategies that adopt this approach. Other phenomena, such as fungal specificity to plant hosts, may also require consideration (Molina *et al.*, 1992). The spectrum of possible plant

hosts that can be selected by a particular mycorrhizal fungus can vary from a few to many. At the same time, the host receptivity (the number of different fungi accepted by a particular plant) can also differ. Both may impact the ecological contribution made by the symbiosis. For example, alder, compared to Douglas-fir, is very selective, typically initiating symbioses with a very restricted number of fungal species. Some fungi may form symbioses with one plant genus, whereas other fungi are less selective, initiating symbioses with potentially hundreds of plant hosts. How this selective nature between fungi and plants impacts ecological functions in general, and bioremediation in particular, remains unknown.

Intrinsic bioremediation (contaminant biodegradation by adapted indigenous communities) may be an acceptable management strategy where risks to human or ecological health are low (Alexander, 1999). Nicolotti and Egli (1998) showed that some ECM fungi surviving in contaminated forest soil may metabolize chemicals in crude oil and suggested that crude oil spills in mixed agricultural and forest areas do not cause long-term environmental damage of the kind associated with coastal ecosystems, possibly due to intrinsic bioremediation by the soil community. Intrinsic bioremediation of PHC-polluted soils differs substantially between ecosystems and depends on the particular combination of soil-forming factors, soil properties, microbial communities and the content and composition of PHCs and their products (Trofimov and Rozanova, 2003). Studies of indigenous microbiota along with key physical and chemical parameters provide hope for the eventual ability to predict how natural attenuation will proceed at contaminated sites. Intrinsic bioremediation may be an appropriate management strategy in boreal forest ecosystems in some situations.

If external action to improve the bioremediation of a soil seems essential, environmental and ecological knowledge is required to choose the least destructive methods (Romantschuk *et al.*, 2000). Ideally, bioremediation strategies should be based on knowledge of the microorganisms present in polluted environments, their metabolic abilities and how they respond to changes in environmental conditions in an ecological context (Blakely *et al.*, 2002; Díaz, 2004). The current state of knowledge, however, does not permit predictions or management strategies to be built up from the species level. Studying the physiology, biochemistry and genetics of soil microorganisms is important for contaminated site bioremediation, as well as for biomonitoring the impacts of chemicals as disturbance agents. Unfortunately, this is mostly unknown and current management is largely based on empirical rather than theory-based deductions. As demonstrated by the research described in this review, factors that alter the survival or activity of soil biota are important considerations for ecosystem management (Setälä *et al.*, 2000). However, the key question in terms of sustainability is how contamination events impact ecosystem functions in the near and long-term future.

Ecosystem health is not well defined, but has been described in terms of vigour (productivity), organization (diversity and mutual dependence) and resilience (maintenance of structures and patterns in the presence of environmental change) and interpreted by correlating biological indicators for processes that are considered critical for ecosystem function (Blakely *et al.*, 2002; Lu and Li, 2003). Traditional indicators for contaminated sites include impacts of chemical contaminants on plant condition and biomass, with more recent interest in specific organisms capable of biodegradation. Fewer studies have

examined changes in soil properties that define its fertility and agro(eco)logical properties (Suleimanov *et al.*, 2005). It has been suggested that criteria for remediation of PHC-contaminated soils should be amended to include suppression or significant modification of the plant community, reduction in plant biomass, disturbance in functioning of soil biota, simplification of the soil community, decreases in the biological activity of the soil, and movement of PHCs into surface water or ground water (Trofimov and Rozanova, 2003). Soil quality guidelines in Canada have been developed using most of these criteria for agricultural, residential/ urban parkland, commercial and industrial lands, but not for forest ecosystems (Ouellet *et al.*, 2002).

Many biological and chemical-physical approaches have been proposed to predict or measure the bioavailability of organic compounds for biodegradation or to ecological receptors (i.e. toxicity). Biological measures of bioavailable toxicants include seedling emergence and growth tests, along with various soil invertebrate tests of acute toxicity, chronic toxicity, behaviour and reproduction (Stephenson *et al.*, 2002). Römbke *et al.* (2006) recently identified potential invertebrate species and testing methodologies for assessing ecotoxicity of contaminants in boreal forest soils. Other biological measures include contaminant uptake and impact on organism biomolecules, and impacts on organism-mediated processes (e.g. nitrification) (Svendsen *et al.*, 2002). Chemical-physical approaches include: kinetics of PHC desorption, mild solvent extraction, solid phase extraction (SPE), supercritical fluid extraction and cyclodextrin extraction. SPE correlates well with biological measures of ecotoxicity (and bioavailability) and does not have the potential to disrupt the structure of soil organic matter phases as do chemical methods (Ehlers and Loibner, 2006).

Conclusions

(1) The importance of developing multi-disciplinary approaches to solving problems relating to anthropogenic pollution is now clearly appreciated by the scientific community, and this is especially evident in boreal ecosystems exposed to escalating threats to PHC contamination through expanded natural resource extraction activities. In this review, we have presented a mycorrhizal ecosystems perspective on PHC contamination in boreal forest soils in order to identify gaps in knowledge and to guide future research in both ecological and sustainable management contexts so that scientists, land and facilities managers, industrialists and government officials will be better prepared to manage the inevitable accidents that will occur.

(2) We know that the taxonomic, genetic and functional diversity of mycorrhizal ecosystems in boreal soils is immense and continues to expand with increased sampling effort. We also know that the functioning of these communities underpins survival and productivity of the ecosystem as a whole. It appears that redundancy in broad-scale biodegradative functions is essential for ecosystem recovery following PHC contamination, to account for the loss of community components that are unable to tolerate the altered physical and chemical conditions imposed by the PHCs. What remains to be determined are the details and the translation of this information into effective ecosystem management.

(3) The ubiquity and enormous biomass of extraradical mycelia of mycorrhizal fungi in forest soils implies a key role in forest ecosystem processes. Recent studies have highlighted

the high taxonomic and genetic diversity of ECM or ERM fungal communities associated with certain plants in some ecosystems, though many potential hosts and types of ecosystems have not yet been surveyed.

(4) Although there is thought to be some relationship between high diversity (species richness) and ecosystem health due to some degree of redundancy, the functional basis of ECM and ERM fungal diversity is virtually unknown. The physiological mechanisms of nutrient exchange between fungal and plant partners are also not well understood, particularly with respect to nutrient acquisition from the soil environment and especially for PHC-contaminated soils. A more thorough knowledge of which fungal symbionts are likely to survive and compete in various ecosystems is required, as well as a better understanding of whether certain types of fungal associations with different plant hosts gain in ecological importance following disturbance events. Whereas community responses (e.g. shifts in community structure) to some types of disturbances (e.g. fire, forestry practices, etc.) have been described in the recent literature (although it is unknown if these are related to shifts in function), responses to PHC pollution are not well understood. In fact, very little is known regarding rhizosphere communities in forest soils subjected to PHC contamination.

(5) Studies of PHC contamination in forest soils are rare, as are the impacts on soil organisms and the intrinsic decomposition in these systems. The scientific basis for current remediation standards is based on information from experiments examining the toxicological impacts of PHC chemicals on test organisms. However, sequestration of organic pollutants in forest SOM may decrease the chemical toxicity of chemicals through physical separation

from biological receptors, which also decreases substrate bioavailability for enzymatic degradation. More research in this area is needed. Also necessary are improved methods for assessing the fate and behaviour of PHCs in forest soils, including determinations of bioavailability and development of a wider variety of indicators for ecological integrity than the traditional measures of plant productivity. Future research is needed to determine how toxicity varies with type of pollutant, mixtures of pollutants, extent of pollution, and the general condition of the ecosystem prior to chemical disturbance.

(6) Few studies have examined whether the coexistence of ECM and ERM plants in boreal forests provides opportunities for sharing ECM and ERM fungi that link plants and translocate nutrients, and virtually nothing is known of how PHC contamination may interfere with processes of nutrient acquisition and exchange.

(7) Recent studies have shown that some ECM and ERM fungi appear to play a direct role (*via* enzymatic catabolism) in biodegradation of complex organic substrates (including PHCs). However, few studies have examined various fungi in detail, or have examined mycorrhizal fungi in symbiosis with a plant. It is unknown as to how PHC contamination might interfere with fungal metabolic processes.

(8) Incomplete biodegradation can produce potentially toxic intermediate metabolites; toxic intermediates with increased water solubility are of particular concern as this can result in the transport and spread of contaminants through the environment. More research is required in this area.

(9) Few studies have considered the indirect role of ECM and ERM systems in biodegradation through their interactions with the mycorrhizosphere-associated bacterial communities and little is known regarding syntrophic biodegradation by different functional guilds of organisms. Most fungi have been examined in isolation from an ecosystem context, thereby excluding interactions of individual ECMs and ERMs with each other, their soil environment and other members of the plant and microbial communities. Thus, information gained from these studies may have little ecological relevance for understanding how forest ecosystems function or for informing bioremediation management strategies for contaminated soils.

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Chapter 2: Interactions between petroleum hydrocarbon contaminants and ecto- and ericoid mycorrhizal communities in sub-boreal forest soils

Abstract

The impacts of petroleum hydrocarbon (PHC) contamination on mycorrhizal communities in boreal forest soils are not well understood. In this study, we used a bioassay approach to determine whether ecologically relevant concentrations of PHCs altered ecto- (ECM) and ericoid (ERM) mycorrhizal fungal communities and whether mycorrhizal communities played a direct role in PHC biodegradation through secretion of laccase. Surface-sterilized seeds (*Pinus contorta*, lodgepole pine; *Betula papyrifera*, paper birch) or seedlings (*Vaccinium vitis-idaea*, lingonberry) were planted into Cone-tainer^{TM} pots containing reconstructed soils: an organic layer (mor humus, coarse woody debris, or previously contaminated humus) overlying sandy mineral horizons (Ae and Bf) of field-collected forest soils obtained from central BC, Canada. After 4 months, BC light crude oil (0, 73, 146, or 219 mg cm^{-2}) was applied to the soil surface around the seedling stem; systems were destructively sampled over 16 weeks following treatment. ECM communities (composition, relative abundance and spatial distribution) were assessed on pine and birch roots using light microscopy and fungal community profiles were generated for all root systems using length heterogeneity PCR and primers targeted at the ITS region of rDNA. In addition, selected mycorrhizal root tips (ECM and ERM) were tested for laccase activity in assays with 2,2'-azinobis-3-ethylbenzthiazoline-6-sulfonate (ABTS). There were no significant changes in ECM or ERM fungal community structure associated with PHC treatment; however, PHC treated systems appeared to exhibit some community differences over time. Plant and organic soil properties appeared to have a greater influence on mycorrhizal community

structure than PHC contamination. In addition, laccase activity was observed for many dominant ECMs, indicating potential for biodegradation of aromatic PHCs. Thus ecological integrity of the plant – soil system may confer resilience against effects of PHC contamination and also contribute to PHC biodegradation.

Introduction

Oil spills that occur in northern forest regions are localized and discrete disturbance events that often result in rapid surface contamination, sometimes with large quantities of petroleum hydrocarbons (PHCs). Very little is known regarding the impacts of PHC contamination on soil communities, particularly on mycorrhizal systems that represent the dominant microbial biomass in forests soils and underpin ecological processes (e.g. decomposition, nutrient cycling, primary production, C sequestration, etc.) both below- and above-ground (Read and Perez-Moreno, 2003). The major impacts of PHC contamination appear to be associated with disturbances to soil water, nutrient and oxygen regimes through changes to physical and chemical properties of soil (Blakely *et al.*, 2002). Changes to soil chemical status and decomposer community functions could lead to imbalances in nutrient cycling and ecosystem productivity (Pennanen *et al.*, 1998). Enhanced hydrophobicity of soils after fire has been shown to alter soil water-holding capacity and lead to changes in both quantity and composition of microbial and soil-dwelling invertebrate communities, potentially reducing plant productivity (Certini, 2005). Thus, factors that alter the survival or activity of mycorrhizal communities are important considerations for sustainable management of forest ecosystems (Setälä *et al.*, 2000). However, this requires that environmental and functional properties assessed at population and community levels be integrated with ecological processes at the ecosystem or landscape scale; this represents a primary challenge of microbial ecology (Bengtsson, 1998; Standing *et al.*, 2007).

In contaminated forests, oil recovery and soil remediation strategies tend to be based on socio-economic considerations and environmental concentrations of some PHCs (e.g.

polycyclic aromatic hydrocarbons, PAHs) with potential toxicity/ carcinogenicity to human and other ecological receptors (Tarradellas and Bitton, 1997; Nicolotti and Egli, 1998; Blakely *et al.*, 2002; Trofimov and Rozanova, 2003). Despite considerable environmental interest in PAHs, a general absence of documented adverse effects among terrestrial receptors (i.e. plants, invertebrates, birds, herpetofauna, or mammals) exposed to PAHs in the environment currently exists in the literature, although this is partly due to lack of research (Kapustka, 2004). Nearly all information regarding PHC toxicity on soil microorganisms has been compiled from laboratory tests that have focused on a few (e.g. PAHs) of the hundreds of chemical compounds that comprise crude oil. Results have generally shown that fungi are more tolerant than bacteria, possibly due to features of cell wall structure (Blakely *et al.*, 2002). However, in addition to reducing the variety and complexity of the biological systems affected by the release of crude oil into the forest environment, these studies nearly always disregard the symbiotic relationship between fungi and plant hosts, which may be very important in terms of buffering negative impacts. Soil-based studies have found that ectomycorrhizal communities exhibit high resilience to environmental stress when organic layers (e.g. humus, woody debris, etc.) of forest soils have not been severely disrupted (Setälä *et al.*, 2000; Jones *et al.*, 2003). Greater environmental disturbance may occur when extreme management measures (e.g. tree or contaminated soil removal) are taken as compared to the original PHC contamination event. The lack of understanding of PHC impacts on mycorrhizal communities in forest soils places remediation strategies in potential conflict with other current forest management objectives.

In situ bioremediation (i.e. enhanced contaminant biodegradation by indigenous soil communities) is considered less destructive and more cost-effective for remediation of contaminated soils on large scales and is also more likely to maintain the desired integrity of mycelial networks (Doelman and Breedveld, 1999). PHC biodegradation is likely due to species-specific fungal attributes that favor survival and degradation activities, extension of the root area in contact with soil-adsorbed compounds, and influence over a complex consortium of bacteria expressing a range of enzyme activities in response to carbon availability in the mycorrhizosphere (Heinonsalo *et al.*, 2000; Meharg and Cairney, 2000). For their part, many ecto- (ECM) and ericoid (ERM) mycorrhizal fungi appear to secrete various hydrolytic and oxidative enzymes that enable direct involvement in the degradation and/ or detoxification of various organic contaminants (Timonen and Sen, 1998; Burke and Cairney, 2002; Perotto *et al.*, 2002). For example, laccase (benzendiol:oxygen oxidoreductase, EC 1.10.3.2), part of a complex, nonspecific enzyme system usually associated with ligninolytic fungi, has been of particular interest with respect to bioremediation (Gramss *et al.*, 1998; Donnelly and Entry, 1999). This enzyme catalyzes the reduction of O_2 to H_2O (4 electron reduction without formation of free reduced oxygen species) to open aromatic ring structures of a range of organic substrates, thereby overcoming the thermodynamically rate-limiting step in microbial metabolic pathways (Burke and Cairney, 2002). In addition to lignin and PAH depolymerization, laccase is thought to be involved in the release of N from insoluble protein-tannin complexes, humus formation, mycelial pigmentation, and fruiting body formation (Kanunfre and Zancan, 1998; Burke and Cairney, 2002). It should be of no surprise that extracellular laccase appears to be constitutively produced in small amounts by several mycorrhizal fungi; it is unknown

whether enzyme expression is enhanced in the presence of different aromatic compounds as appears to be the case for some saprotrophic fungi (Burke and Cairney, 2002).

This study addresses a fundamental question: does PHC contamination of forest soils negatively impact established (or establishing) mycorrhizal systems? Using a bioassay (single- and double-plant systems established in reconstructed forest soil layers in Cone-tainer™ pots), morphological and molecular (LH-PCR) techniques were used to assess changes in mycorrhizal community structure in response to different PHC concentrations over 16 weeks. In these systems, organic soil layers (i.e. forest floor [FH], coarse woody debris [CWD], and forest floor previously contaminated with PHCs [FHoil]) provided the initial fungal inoculum for the developing rhizospheres; the composition of fungal communities was expected to be influenced by the relative abundance of lignin (i.e. in FH and CWD) and/ or previous exposure to PHCs (i.e. in FHoil). ECM (*Pinus contorta* var. *latifolia* and *Betula papyrifera*) and ERM (*Vaccinium vitis-idaea*) systems were expected to represent a sub-set of the mycorrhizal fungal community *in situ*. Laccase assays were also conducted to assess potential for direct role of ECM and ERM fungi in PHC biodegradation and assess changes in enzyme activity associated with PHC treatment.

Materials and methods

Field site

The Kenneth Creek field site is located in the wet, cool subzone of the sub-boreal spruce (SBSwk1) biogeoclimatic zone of central British Columbia, Canada, about 100 km east of Prince George (53°34'N, 122°47'W) (Figure 2.1). In 1982, the forest was logged and burned, then subsequently planted with lodgepole pine (*Pinus contorta* Dougl. Ex Loud. var. *latifolia* Engelm.); currently, the site is a mature, even-aged pine stand with small hybrid white spruce (*Picea glauca* x *engelmannii* Parry *ex* Engelm.) and lesser numbers of western hemlock (*Tsuga heterophylla* (Raf.) Sarg.). Young subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.) and sitka alder (*Alnus crispa* var. *sinuata* (Reg.) Rydb.) are present at the edge of the forest, along the access road; western redcedar (*Thuja plicata* Donn) and trembling aspen (*Populus tremuloides* Michx.) are also present in an unlogged stand across the main road. The site has a thick understory of oval-leaved blueberry (*Vaccinium ovalifolium* Sm.); mosses and lichens (e.g. *Peltigera*) cover the forest floor, with some *Lycopodium* species.

The soils on this site were described by Arocena and Sanborn (1999). Soils are classified as Eluviated Dystric Brunisols (Soil Classification Working Group, 1998), and consist of sandy parent material with low clay content and few coarse fragments. The forest floor is mor humus, from 2-5 cm thick with copious fungal mycelia present. The C:N ratio of the forest floor is approximately 50 and the pH (water) is ~ 4.2. Gray Ae horizons are generally 1-2 cm thick, with thicker pockets in some areas. Red Bf horizons extend to almost 30 cm, beneath which are Bm (27-60 cm), BC (60-100) and C (> 1 m) horizons. The C:N ratios of the Ae and Bf horizons are about 20 and 13, respectively. The pH (water) of the Ae horizon is 4.2

while the pH of the Bf horizon is about 4.8, increasing with depth to about 6.0 at the transition to the C horizon. Fine roots are found at depths greater than 1 m. Large coarse woody debris (i.e. downed trees), the legacy of past forest management, is abundant all over the site.

Organic layers and the top 20-30 cm of mineral soils (Ae and Bf horizons) were collected from the forest site in September of 2004, 2005 and 2006. The organic layers included the mor humus forest floor (FH) that had been undisturbed for approximately 20 years, coarse woody debris (CWD) in an advanced state of decay (i.e. decay class 5), and previously PHC-contaminated mor humus (FHoil) that had weathered *in situ* for four months throughout the summer. The FHoil soils were collected in 2005 (year 2) only. In early May of that year, 2.0 L of BC light crude oil (Husky Refinery, Prince George, BC) was applied to each of three 1-m^2 plots with the moss layer (but not seedlings and other plants) removed. The oil had been previously bubbled with N_2 gas to remove the light and volatile compounds; a watering can was used for even application in the field. All soils were stored at 4°C prior to use in bioassay experiments, which commenced within 10 days of soil collection.

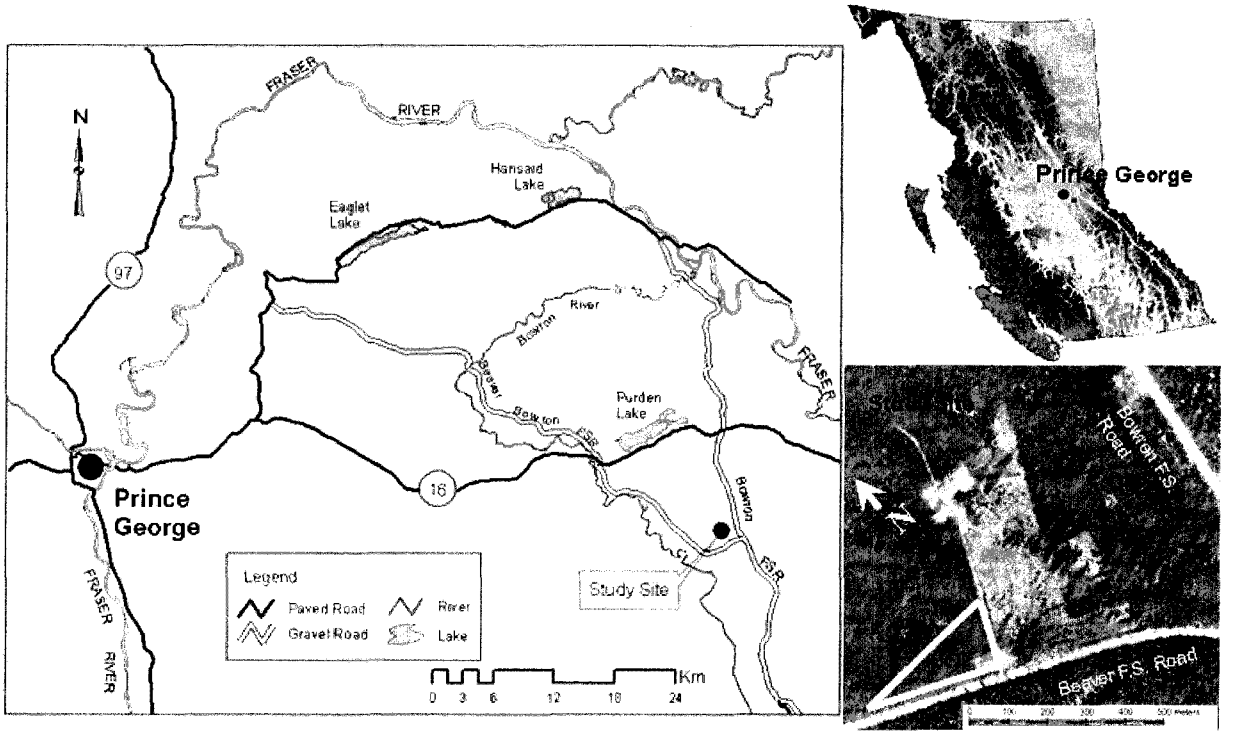

Figure 2.1: Maps showing the study site at the Kenneth Creek field site, located about 100 km east of Prince George in the sub-boreal spruce (SBSwk1) zone of the central interior of British Columbia, Canada.

Bioassay and PHC treatment

Forest soils (i.e. Ae [~1 cm] and Bf [~15 cm] mineral soil layers beneath organic [FH, CWD, or FHoil] soil layers [~2 cm]) were reconstructed in Cone-tainer™ pots (3.8 x 21 cm, Stuewe and Sons, Corvallis, Oregon) with two clay pellets in the bottom to prevent soil loss (Setälä *et al.,* 2000). Mineral soils (Ae and Bf layers) were homogenized and sieved through (1 cm^2) screens prior to potting. In 2004 and 2005 (years 1 and 2), surface-sterilized seeds of lodgepole pine (*Pinus contorta* var. *latifolia*) and paper birch (*Betula papyrifera* Marsh.), collected from the SBS and obtained from the Ministry Tree Seed Center, Surrey, BC (Seed lots DWD20050009A (location 079-B-008) and DWD20050009B (location 094-E-015), respectively), were planted into each pot. In 2005 (year 2), lingonberry (*Vaccinium vitis-idaea* L.) seedlings (rooted cuttings), obtained from Birch Creek Nursery (Prince George, BC), were planted into 10x10x10 cm pots (i.e. Ae [~1 cm] and Bf [~7 cm] mineral soil layers beneath organic [~2 cm] soil layers). In 2006 (year 3), pine seeds were planted into Cone-tainer™ soil systems (FH and CWD organic layers) that were already planted with lingonberry cuttings. All pots were placed in the greenhouse (22°C day temperature, 15°C night temperature, and 16 h photoperiod) and fertilized once a month (5 mL of NPK fertilizer; providing 100 ppm each of NPK) for the first four months following seeding/ planting. The plants were watered two or three times per week for the duration of the experiment.

After four months of growth, seedlings and mycorrhizas were well established. At this time, BC light crude oil (with volatiles removed as described for the field application) was pipetted onto the organic soil surface of each pot, around, but not touching, the seedling stem(s). To

determine effects of different levels of PHC contamination on plant – soil systems, 1, 2, or 3 mL (corresponding to 73, 146, and 219 mg cm^{-2}, respectively) crude oil was tested on pine and birch seedlings in 2004-05 (year 1). In the two subsequent years of study (i.e. years 2 and 3), pine, birch, and lingonberry seedlings were treated with the highest (219 mg cm^{-2}) PHC level tested, which corresponded to a field application rate of nearly 22 tonnes ha^{-1}. There was no PHC loss from the bottom of the pot (i.e. no sheen on the wet surface below) observed after watering for the duration of the experiment and the smell of crude oil dissipated in the greenhouse within a week of PHC treatment. These observations provided some confidence that most of the oil was retained within the plant – soil systems (i.e. not lost through volatilization or washing out).

Experimental design and sampling

Experiments followed a randomized block design and tested different combinations of plant, organic soil layer and PHC treatments (Tables 2.1 and 2.2) over three years of studies. In 2004-05 (year 1), treatment combinations included plant (P or B) and organic layer (FH or CWD) systems in PHC contaminated and untreated (control) soils (Table 2.1). Plant – soil systems (n=2 per treatment group) were destructively sampled at 1, 4, 7, 10, 13 and 16 weeks following PHC treatment in order to assess ECM morphotype community changes over time in response to different levels (0, 73, 146, and 219 mg cm^{-2}) of contamination. Single-plant (pine, birch, and lingonberry) and double-plant (pine and lingonberry) systems (2005-06 and 2006-07, respectively) were harvested at 1 and 16 weeks to further assess relationships between ECM and ERM fungal communities and PHC biodegradation (Table 2.2).

Table 2.1: Summary of plant, organic soil layer, and PHC treatment variables for 2004-2005 study (n=2).

Plant	Organic Soil Layer	PHC
Pine [P]	Forest floor [FH]	No PHC (control)
Birch [B]	Coarse woody debris [CWD]	PHC (73 mg cm^{-2})
		PHC (146 mg cm^{-2})
		PHC (219 mg cm^{-2})

Table 2.2: Summary of plant, organic soil layer, and PHC treatment variables for 2005-2006 (single-plant) and 2006-2007 (double-plant) studies (n=3)*.

Plant	Organic Soil Layer	PHC
Pine [P]	Forest floor [FH]	No PHC (control)
Birch [B]	Coarse woody debris [CWD]	PHC (219 mg cm^{-2})
Lingonberry [L]	Contaminated forest floor [FHoil]	
Pine + Lingonberry [P+L]		

* n<3 in B-CWD and B-FHoil groups due to lack of seed germination

Root systems (intact, but with the shoot excised) were shaken free from the soil (samples for PHC and nutrient analysis, pH and wet weights were collected concurrently, as described in Chapter 3) and then washed in sterile dH_2O. A detailed morphological analysis of pine and birch ECMs was conducted in 2004-05 (year 1). For this, root systems were stored in glass plates with sterile water at 4°C for up to a week following each harvest. Molecular analysis of ECM/ ERM fungi was conducted in 2005-06 (year 2) for the single-plant systems and in 2006-07 (year 3) for the double-plant systems. Within a few days of seedling harvest, pine and birch root systems were quickly assessed under a dissecting microscope to note ECM presence, abundance and distribution within the soil system and to ensure that they were free of other roots or hyphae and soil particles. Samples of lingonberry hair roots were assessed for cortical cell colonization by root endophytes (i.e. cells appeared cloudy, not clear), some of which were expected to be ERM fungi. Except for a few root tips removed for laccase

assays, which were also performed at the 1 and 16-week harvest times, entire root systems were then collected in 2 mL tubes and stored at -20°C until DNA extraction.

Morphological assessment of ECM communities

Standard light microscopy techniques of Agerer (1987-2002), Ingleby *et al.* (1990) and Goodman *et al.* (1996) were used to group, quantify and spatially describe ECM morphotypes of PHC treated and untreated (control) pine and birch seedlings at 3-week intervals over the 16-week duration of the experiment. ECMs were initially described and grouped according to colour, texture, lustre, dimensions, tip shape, branching pattern, and presence or absence of rhizomorphs (mycelial strands). Root squash mounts were examined at 400-1000X magnification and descriptions of mantle features, emanating hyphae, rhizomorphs, and other distinguishing features were used to further group different ECM morphotypes. Root tips that appeared uncolonized or partially colonized (due to the lack of a well-developed mantle) were categorized as non-mycorrhizal. When possible, preliminary identifications to fungal families or genera were assigned; otherwise, a descriptive name was assigned.

ECM community diversity was represented by the Simpson diversity index, a non-parametric measure of relative abundance of each ECM morphotype weighted more towards the abundant types (Magurran, 2004). Simpson diversity (D) was calculated for each root system from $D = \Sigma p_i^2$, where p_i represents the proportion of individuals in the i^{th} species, and reported as 1/D. Diversity values were compared using one-way ANOVA to determine significant differences (α=0.05) with level of PHC treatment, over time, and between the

plant – soil systems. The post-hoc Fisher's LSD test (α=0.05) was performed when a significant result was obtained.

DNA extraction and length heterogeneity PCR

Frozen root systems were crushed in liquid nitrogen and DNA was extracted using a CTAB (hexadecyl trimethyl ammonium bromide) protocol with an extra phenol/chloroform-isoamyl alcohol (1:1) purification step (Fujimura *et al.*, 2008). DNA extracts were further cleaned using the Wizard® PCR Preps DNA Purification System kit (Promega) to remove phenolics and other oily contaminants. The cleaned DNA extracts were resuspended in TE buffer.

Fungal communities were characterized by amplicon length heterogeneity PCR (LH-PCR), which provides an estimate of community structure based on relative abundance of genotypes (Martin and Rygiewicz, 2005). Community fingerprinting methods such as LH-PCR and terminal restriction fragment length polymorphism (TRFLP) provide little taxonomic resolution of microbial communities compared to methods such as sequencing, but are expected to provide sufficient resolution to separate communities based on broad variables (Kuyper and Landeweert, 2002). The ITS2 region of ribosomal DNA was amplified using the forward primer ITS3 (5'GCATCGATGAAGAACGCAGC) (White *et al.*, 1990) and the D3 fluorescent dye-labeled reverse primer NL4B (5'GGATTCTCACCCTCTATGAC) (Martin and Rygiewicz, 2005). ITS3 is a universal primer that binds in a conserved domain 128 bp from the 3' end of the 5.8S rDNA while NL4B binds in the large subunit (28S) at basidiomycete- (and ascomycete-) specific sites (Martin and Rygiewicz, 2005); PCR products are expected to vary from approximately 400 to >600 bp in length. PCR reactions

consisted of 10X PCR buffer, 2 mM dNTPs, 50 μM $MgCl_2$, 10 μM forward and reverse primers (Proligo, CO), 0.7 U Platinum Taq DNA polymerase (Invitrogen Life Technologies), and nuclease-free water (Integrated DNA Technologies, Inc.) to a final volume of 27 μL, to which 3 μL DNA (diluted 1:10) was added. The DNA Engine DYAD™ thermocycler (MJ Research, Inc., Watertown, MA) conditions were as follows: initial denaturation for 4 min at 94°C, annealing for 1 min at 48°C, and extension for 2 min, followed by 35 cycles of denaturation (94°C for 30 s), annealing (48°C for 30 s) and extension (72°C for 1 min 30 s) and final extension at 72°C for 6 min 30 s. All PCR products were run on 1.2% agarose gels to confirm amplification.

PCR products (2 μL) were loaded into a CEQ™ 8000 sequencer (Beckman-Coulter Inc.) along with CEQ 600 bp size standard mixture. Run conditions were 60°C separation temperature, 4 kV voltage, and 120 min separation time. Analysis was performed using the amplicon fragment length polymorphism (AFLP) program of the CEQ™ 8000 sequencer and the quartic model for size standard with a bin width of 1.5 bp. Peaks less than 11% of the total sample peak height were not included. Profiles from separate DNA extractions and PCR reactions were compared to assess reproducibility and suitability for analysis.

The relative abundance of genotypes comprising each sample (i.e. fungal community) was calculated after relativizing the fluorescent signal strength of each fragment peak to the total peak area within each sample. This step in the analysis set the minimum peak threshold by removing the small peaks resulting from noise or reflecting the amount of DNA analyzed that may impact the conclusions drawn (Osborne *et al.*, 2006).

Ordination techniques are used to arrange entities along single or multiple axes that summarize the continuous trend within data; samples with similar community composition or environmental characteristics are grouped more closely in the ordination space (McCune and Grace, 2002). Nonmetric Multidimensional Scaling (NMS) is a preferred method for analysis of community data because it does not carry assumptions of linearity among variables (required for parametric approaches) (Ramette, 2007). NMS preserves similarity distances in ranked order (i.e. nonparametric) and tends to linearize distances in species and environmental space; it is not constrained to any specific distance measure. In this study, NMS was calculated on the basis of a Sørensen distance measure with 50 runs with real and randomized data and a maximum of 500 iterations to assess stability (instability criterion was 0.00001) using PC-ORD 5.0 software (McCune and Mefford, 1999). A stepwise reduction in dimensionality (6D-1D) was used to minimize stress along with a random starting configuration (user-provided seeds). Two measures were used to evaluate the structure of the ordination results. Stress (i.e. goodness of fit measure) is the deviation from monotonicity when distance is compared between the original species space and distance in the reduced ordination space (McCune and Grace, 2002). For community data, it is typically in the 10-20 range, but should be interpreted cautiously at the upper end of this scale. Instability is a measure of change in stress at each iteration. Stable, low stress solutions indicate strong data structure. The final solution for NMS was accepted after comparing 50 runs with real to randomized data using Monte Carlo simulations (McCune and Grace, 2002).

Univariate comparisons between treatment groups were tested statistically with Multi-Response Permutation Procedures (MRPP), a non-parametric method that tests the hypothesis that there is no difference among groups (McCune and Grace, 2002). It provides a statistic of the magnitude of differences between groups (analogous to effect size), given as the chance-corrected, within group agreement (A) and a p-value. A is equal to 1 when all items are identical within groups and 0 when heterogeneity within groups equals expectation by chance. As most comparisons were made between unequal group sizes (i.e. unbalanced analyses), multivariate differences were not tested statistically with nested permutational multivariate ANOVA, and thus direct interaction effects could not be detected.

Laccase assays

Laccase assays were conducted in 2006 and 2007 (years 2 and 3) with pine and birch (ECM) root tips and lingonberry (ERM) hair roots. Laccase activity was determined using a plate assay with 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonate (ABTS; Sigma Chemical Co., St. Louis, Mo.) as the substrate (Eggert *et al.* 1996). Freshly harvested plant roots were viewed under a dissecting microscope to select ECM morphotypes and to confirm the presence of colonized epidermal cells in ERM hair roots. These were removed from the root systems and placed into individual wells of a microplate, each well containing 100 µL of 50 mM glycine-HCl buffer (pH 3). Following addition of 50 µL ABTS (2 mM) to each well, the plates were incubated at ambient temperature (~22°C) for 24 h. The intensity of the bluish green color development was rated as nil (-), light (+) or dark (++) after 1, 16 and 24 h of incubation. In an attempt to exclude reactions mediated by other potentially-present oxidative enzymes, assays were conducted at pH 3, the optimum pH for laccase activity in the white rot fungus,

P. chrysosporium with ABTS as the substrate; peroxidase reactions with ABTS generally occurred at pH 4-7. A small piece of field-collected *Trametes* fungus (collected behind the EFL at UNBC) was used as a positive control (dark green colour developed within an hour of incubation) for this assay; boiled fungus was used as the negative control (no colour development after several days).

Results

ECM morphotypes: development and response to PHCs

All seedlings and root systems survived for 16 weeks following PHC treatment. A greater proportion of seedlings generally appeared less healthy (i.e. yellowed or red needles/ leaves) with the highest PHC treatment compared to controls, but untreated plants also occasionally appeared chlorotic. There was no difference in seedling height or root system development between control and PHC treated groups (data not shown).

Virtually all pine and birch root tips were visibly ectomycorrhizal at the first harvest time (i.e. approximately 4 months post-seed germination and 1 week post-PHC treatment). The richness of ECMs associated with each root system ranged from two to seven morphotypes, with fewer distinct morphotypes observed on birch compared to pine roots. Morphotype richness tended to increase over time (i.e. 1-16 weeks) for both plant species and organic soil types. In general, ECMs, extraradical mycelia, and rhizomorphs were more extensively developed by the 16-week sampling time, which may have contributed to their greater visibility. Differences between pine and birch root system structures influenced the distribution of ECMs through the soil system, as well as ECM density within the pot and

proximity to other root tips. Pine root tip density was greater in the organic layer of the soil profile, but more root tips (i.e. up to 85%) occurred in the greater volume of the Bf layer. Pine generally had a lower density of root tips compared to birch.

Morphotype descriptions for pine and birch ECMs are provided in Appendix A. ECM morphotypes that were most frequently identified on root systems included *Cenococcum*, *MRA*, E-strain, *Amphinema*, three Russulaceae (including *Lactarius*), two *Rhizopogon-Suillus*, and two Thelephoraceae types. Three unidentified types (one black and two white types) were observed less commonly. The relative abundance of ECMs in FH and CWD soil systems are shown for pine and birch in Figures 2.2 and 2.3, respectively.

Clusters of *Cenococcum* ECMs dominated the upper roots of pine and birch in the FH layer (i.e. often representing 10-20% of the total root tip community), but were rarely observed in the CWD layer. These tips were black and woolly, with characteristic stellate pattern of cells of the outer mantle. Although relative abundance did not decrease in systems with higher PHC concentrations, root tips appeared dry and less robust over time with PHC treatment. The other ascomycetes, *MRA* and E-strain, were commonly found on root tips throughout the soil profile, but with low relative abundance. All three ascomycetes were well-developed at the 1-week sampling times; at later sampling times, evidence of secondary colonization was observed, in which new ECM growth occurred on root tips previously colonized by *MRA*.

Amphinema ECMs and external mycelia were often associated with *Cenococcum* tips near the organic-Ae layer interface, but were also occasionally observed lower in the soil profile.

Amphinema was characterized by copious yellow, curvy, clamped hyphae, but no rhizomorphs were observed in this study. *Piloderma* hyphae, characterized by ornamentation with needle-like crystals, were found associated with *Cenococcum* tips, but were never observed as ECMs in this study, even though abundant *Piloderma* mycelia were observed in the FH, CWD and FHoil organic layers in the field.

Russulaceae types dominated most root systems (i.e. ECMs observed on 80-90% of root tips) of both pine and birch in FH and CWD soil systems. These ECMs were developed by the 1-week harvest time, although sometimes with a thin mantle. Two Russulaceae types were associated with pine. Root tips varied in color from yellow to orange-brown and were generally unbranched and smooth or appeared velvety due to the few short unclamped hyphae that resembled cystidia early in their development. The cell arrangement of outer mantles ranged from net to irregular synenchyma. The Russulaceae 2 ECM was identified as a species of *Lactarius* based on the presence of laticifers, which contained latex material. On birch, the Russulaceae 3 ECM, a cream-coloured smooth type, dominated all 96 root systems assessed in the first year of the study. Although mainly found in the mineral soil layers, Russulaceae ECMs were also common on the upper lateral roots, particularly in the CWD organic layer.

Two *Rhizopogon-Suillus* types were found associated with root tips exclusively in the Bf layer in FH and CWD soil systems associated with pine. The *Rhizopogon-Suillus* 1 ECM was a dark brown, coralloid morphotype. The outer mantle had a net synenchyma cell arrangement with amorphous globules at the surface; the copious hyphae (2-4 μm)

characteristically bulged at the unclamped septa and exhibited no other ornamentation. The *Rhizopogon-Suillus* 2 ECM was white with a slightly tuberculate form, copious hyphae and an outer mantle with prosenchymous (to net synenchymous) cell arrangement. By the 16-week sampling time, both ECMs had developed extensive rhizomorphs and hyphal fans (stained purple and orange, respectively, with KOH) that extended away from root tips into the mineral soil. Although ECM/ mycelial biomass was not measured, it appeared that rhizomorphic development may have been enhanced in PHC contaminated soils.

Two Thelephoraceae -like types were found associated with a few seedlings of pine and birch in both FH and CWD soil systems. These ECMs were never observed prior to the 7-week harvest and were present at all concentrations of PHCs. ECMs were smooth, ranged in colour from orange to dark brown or black and had thin mantles of net or regular synenchyma (respectively). Both had few long emanating hyphae, sometimes with clamps.

Three ECMs, one black and two white, were not identified in this study, mainly because they were encountered so rarely. The black type resembled *MRA*, except that the outer mantle was net to irregular synenchyma rather than the felt prosenchyma typically associated with *MRA*. One white type was similar to *Piloderma* and was found exclusively with birch.

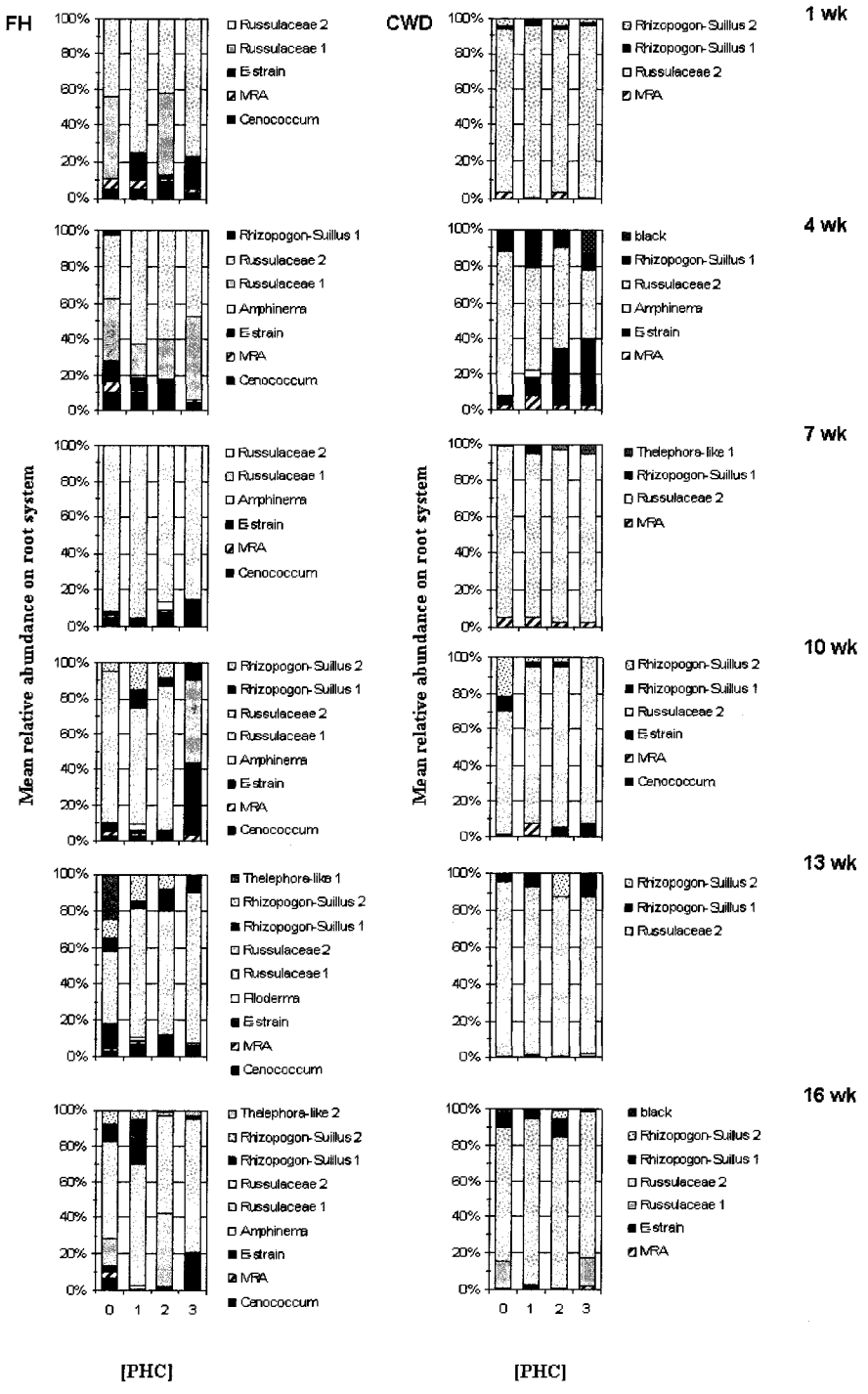

Figure 2.2: Relative abundance of ECMs on pine roots in FH and CWD soil systems sampled at 3-week intervals over 16 weeks with 0, 1, 2, or 3 mL of crude oil added to the soil surface.

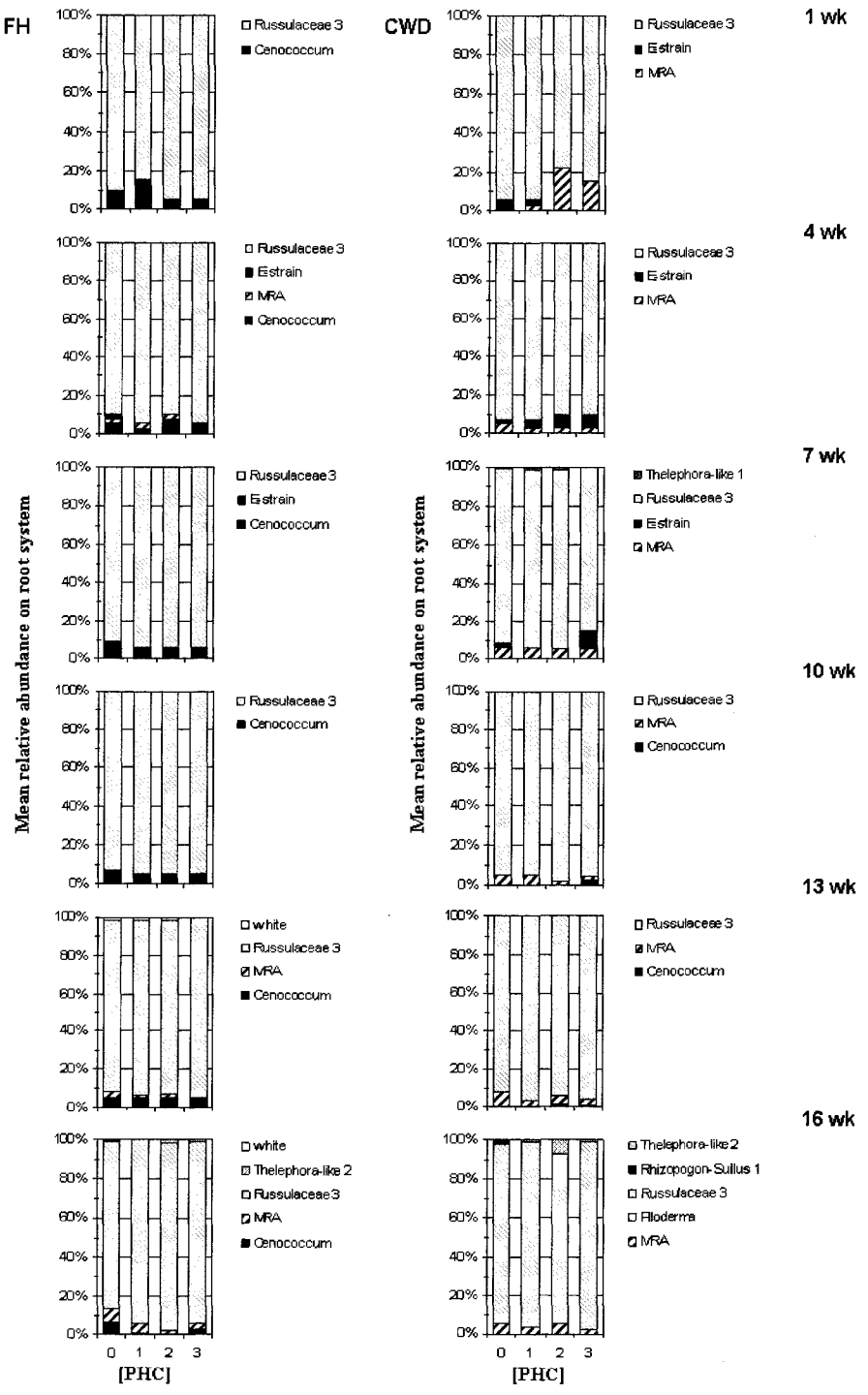

Figure 2.3: Relative abundance of ECMs on birch roots in FH and CWD soil systems sampled at 3-week intervals over 16 weeks with 0, 1, 2, or 3 mL of crude oil added to the soil surface.

In general, Simpson diversity (1/D) values were lower in systems treated with the highest PHC concentration (219 mg cm^{-2}) compared to untreated controls, with intermediate diversity values for the intermediate (i.e. 73 mg cm^{-2} and 146 mg cm^{-2}) PHC concentrations (Table 2.3). Differences between PHC treatments within harvest groups (n=2) were significant for P-FH systems at 10 weeks (p=0.027) and for B-FH systems at 13 and 16 weeks (p = 0.015)

Table 2.3: One-way ANOVA plus Fisher's LSD ($\alpha = 0.05$) of Simpson diversity (1/D) between PHC treatments (within harvest groups): PHC-0 = control; PHC-1 = 73 mg cm^{-2}; PHC-2 = 146 mg cm^{-2}; PHC-3 = 219 mg cm^{-2}.

Harvest	PHC-0 mean (SE)	PHC-1 mean (SE)	PHC-2 mean (SE)	PHC-3 mean (SE)
P-FH				
1 wk	1.23 (0.39)	1.65 (0.37)	1.29 (0.27)	1.58 (0.36)
4 wk	1.94 (0.39)	2.33 (0.37)	2.40 (0.27)	1.13 (0.36)
7 wk	1.19 (0.39)	1.11 (0.37)	1.35 (0.27)	1.39 (0.36)
10 wk	1.36 (0.39) *a*	2.11 (0.37) *ab*	1.52 (0.27) *ab*	2.44 (0.36) *b*
13 wk	3.07 (0.39)	1.77 (0.37)	1.99 (0.27)	1.49 (0.36)
16 wk	2.80 (0.39)	1.84 (0.37)	2.16 (0.27)	1.54 (0.36)
P-CWD				
1 wk	1.23 (0.33)	1.11 (0.21)	1.24 (0.27)	1.11 (0.22)
4 wk	1.52 (0.33)	2.46 (0.21)	2.07 (0.27)	2.35 (0.22)
7 wk	1.11 (0.33)	1.23 (0.21)	1.10 (0.27)	1.17 (0.22)
10 wk	2.01 (0.33)	1.3 (0.21)	1.24 (0.27)	1.17 (0.22)
13 wk	1.12 (0.33)	1.20 (0.21)	1.33 (0.27)	1.36 (0.22)
16 wk	1.80 (0.33)	1.19 (0.21)	1.41 (0.27)	1.49 (0.22)
B-FH				
1 wk	1.24 (0.10)	1.35 (0.11)	1.10 (0.02)	1.10 (0.00)
4 wk	1.23 (0.10)	1.11 (0.11)	1.22 (0.02)	1.10 (0.00)
7 wk	1.22 (0.10)	1.13 (0.11)	1.22 (0.02)	1.10 (0.00)
10 wk	1.1 6 (0.10)	1.12 (0.11)	1.12 (0.02)	1.10 (0.00)
13 wk	1.21 (0.10) *a*	1.17 (0.11) *ab*	1.18 (0.02) *ab*	1.12 (0.00) *b*
16 wk	1.36 (0.10) *a*	1.12 (0.11) *b*	1.08 (0.02) *c*	1.15 (0.00) *d*
B-CWD				
1 wk	1.12 (0.07)	1.11 (0.08)	1.53 (0.11)	1.34 (0.02)
4 wk	1.17 (0.07)	1.16 (0.08)	1.22 (0.11)	1.22 (0.02)
7 wk	1.12 (0.07)	1.11 (0.08)	1.53 (0.11)	1.36 (0.02)
10 wk	1.10 (0.07)	1.1 (0.08)	1.04 (0.11)	1.10 (0.02)
13 wk	1.16 (0.07)	1.07 (0.08)	1.13 (0.11)	1.08 (0.02)
16 wk	1.17 (0.07)	1.09 (0.08)	1.31 (0.11)	1.07 (0.02)

Analysis of Simpson diversity values within PHC groups generally showed an increase over the 16-week duration of the experiment (Table 2.4). These differences were significant within the two higher PHC concentration groups (i.e. 146 mg cm^{-2} and 219 mg cm^{-2}) for P-FH ($p=0.004$), B-FH ($p=0.002$), and B-CWD ($p=0.013$) systems, as well as within the untreated control group for P-FH ($p=0.004$).

Table 2.4: One-way ANOVA plus Fisher's LSD ($\alpha = 0.05$) of Simpson diversity (1/D) between harvests (within PHC treatment groups) at 1, 4, 7, 10, 13, and 16 weeks following PHC treatment.

Harvest	PHC-0 mean (SE)	PHC-1 mean (SE)	PHC-2 mean (SE)	PHC-3 mean (SE)
P-FH				
1 wk	1.23 (0.39) *a*	1.65 (0.37)	1.29 (0.27) *a*	1.58 (0.36) *ab*
4 wk	1.94 (0.39) *ab*	2.33 (0.37)	2.40 (0.27) *b*	1.13 (0.36) *a*
7 wk	1.19 (0.39) *a*	1.11 (0.37)	1.35 (0.27) *a*	1.39 (0.36) *ab*
10 wk	1.36 (0.39) *a*	2.11 (0.37)	1.52 (0.27) a*b*	2.44 (0.36) *b*
13 wk	3.07 (0.39) *b*	1.77 (0.37)	1.99 (0.27) *ab*	1.49 (0.36) *ab*
16 wk	2.80 (0.39) *b*	1.84 (0.37)	2.16 (0.27) *ab*	1.54 (0.36) *ab*
P-CWD				
1 wk	1.23 (0.33)	1.11 (0.21)	1.24 (0.27)	1.11 (0.22)
4 wk	1.52 (0.33)	2.46 (0.21)	2.07 (0.27)	2.35 (0.22)
7 wk	1.11 (0.33)	1.23 (0.21)	1.10 (0.27)	1.17 (0.22)
10 wk	2.01 (0.33)	1.3 (0.21)	1.24 (0.27)	1.17 (0.22)
13 wk	1.12 (0.33)	1.20 (0.21)	1.33 (0.27)	1.36 (0.22)
16 wk	1.80 (0.33)	1.19 (0.21)	1.41 (0.27)	1.49 (0.22)
B-FH				
1 wk	1.24 (0.10)	1.35 (0.11)	1.10 (0.02) *ad*	1.10 (0.00) *a*
4 wk	1.23 (0.10)	1.11 (0.11)	1.22 (0.02) *b*	1.10 (0.00) *a*
7 wk	1.22 (0.10)	1.13 (0.11)	1.22 (0.02) *ab*	1.10 (0.00) *a*
10 wk	1.1 6 (0.10)	1.12 (0.11)	1.12 (0.02) *c*	1.10 (0.00) *a*
13 wk	1.21 (0.10)	1.17 (0.11)	1.18 (0.02) *d*	1.12 (0.00) *a*
16 wk	1.36 (0.10)	1.12 (0.11)	1.08 (0.02) *ae*	1.15 (0.00) *b*
B-CWD				
1 wk	1.12 (0.07)	1.11 (0.08)	1.53 (0.11) *a*	1.34 (0.02) *a*
4 wk	1.17 (0.07)	1.16 (0.08)	1.22 (0.11) *ab*	1.22 (0.02) *b*
7 wk	1.12 (0.07)	1.11 (0.08)	1.53 (0.11) *a*	1.36 (0.02) *a*
10 wk	1.10 (0.07)	1.1 (0.08)	1.04 (0.11) *b*	1.10 (0.02) *c*
13 wk	1.16 (0.07)	1.07 (0.08)	1.13 (0.11) *b*	1.08 (0.02) *c*
16 wk	1.17 (0.07)	1.09 (0.08)	1.31 (0.11) *ab*	1.07 (0.02) *c*

ECM and ERM community structure

Multivariate analysis using nonmetric multidimensional scaling (NMS) gave a three-dimensional solution with a final stress of 19.66 and instability of 0.12. The NMS ordination in Figures 2.4 and 2.6 show relative fungal community structure (genotypes) for all 109 plant-soil systems for which successful fragment analysis occurred. These included 24 pine, 12 birch, and 25 lingonberry single-plant systems, as well as 24 pine and lingonberry double-plant systems in 48 FH, 43 CWD and 18 FHoil (used in single-plant systems only) soils. Nearly half of all systems were treated with PHCs (53 treated and 56 untreated systems); 54 and 55 systems, respectively, were harvested from 1 and 16 weeks. Except for birch –CWD and FHoil groups, all other (plant-soil-PHC-harvest) treatment groups were represented by n=2-3. The total fungal community was represented by 91 DNA fragment lengths (genotypes): 74 were associated with pine and 60 with lingonberry (3 genotypes were exclusive to birch). Thirty genotypes were associated with double-plant systems only, 30 were associated with only single-plant systems, and 31 genotypes were common to both single- and double-plant systems.

Harvest time and PHC effects

NMS ordinations of the harvest time and PHC treatment variables are shown along the first and second axes of Figure 2.4. Overall, fungal community structure varied significantly ($p=0.005$) between the 1 and 16-week harvest times. PHC treatment was not found to lead to any significant differences in fungal community structure when considered as a single variable. However, comparisons within PHC treated and control groups revealed that the PHC treated group at the 16-week harvest significantly varied from both PHC treated

(p=0.005) and control (p=0.031) groups at the 1-week harvest, while fungal communities in the control group did not vary after 16 weeks (Figure 2.5). In Figure 2.4, a great deal of overlap is observed in the distribution of fungal communities at the two harvest times (represented by diamond and inverted triangle symbols, respectively), but the 16-week group appears to be more concentrated towards the upper left quadrant of the plot. The separation of PHC treated communities (closed symbols) should appear more pronounced than untreated control communities (open symbols).

ECM fungal communities of pine exhibited the same trends observed for the overall fungal community; namely, that PHC impacts on fungal community structure were observed only over time (p=0.031). ERM communities of lingonberry communities showed no differences between PHC treatment and harvest time groups.

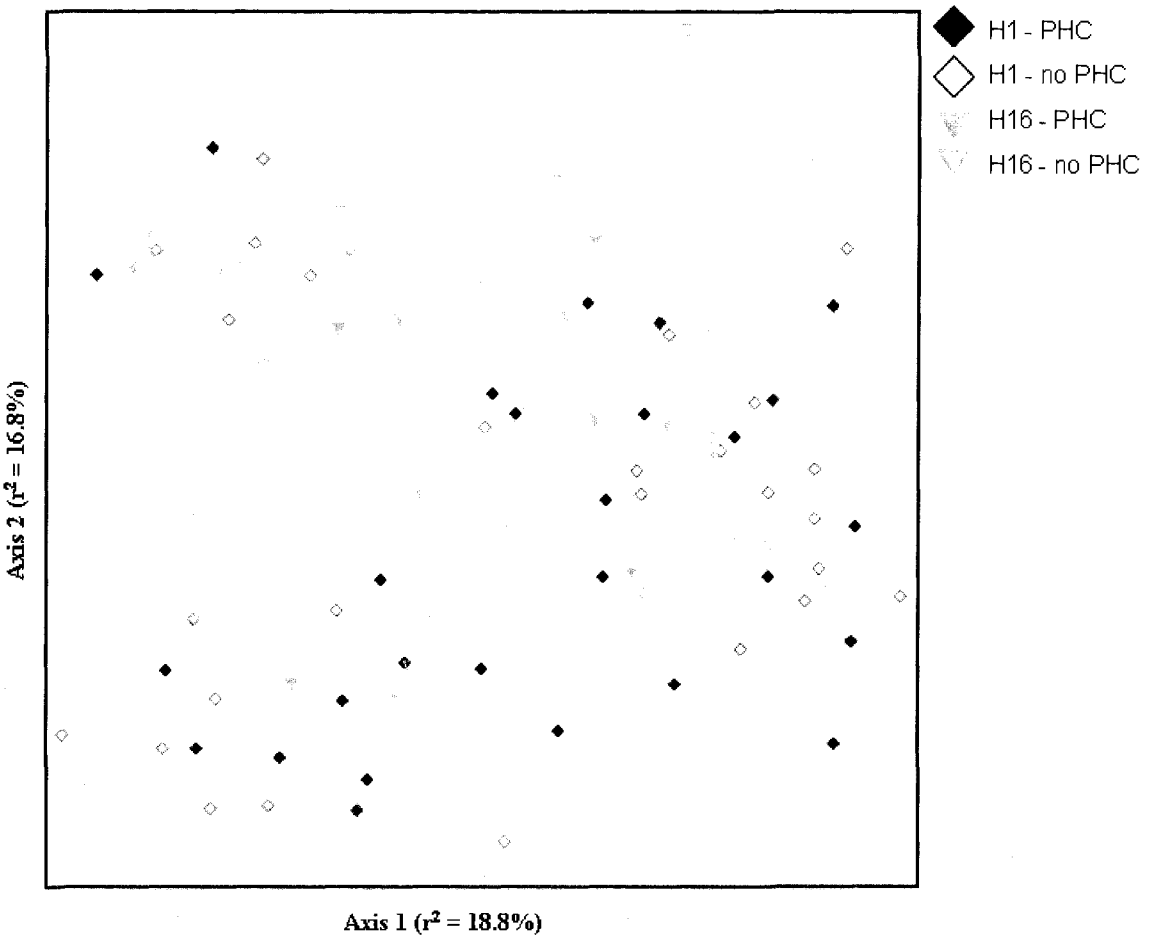

Figure 2.4: NMS ordination of mycorrhizal fungal showing multivariate effects of harvest time (1 and 16 weeks) and PHC treatment (stress = 19.66; instability = 0.12).

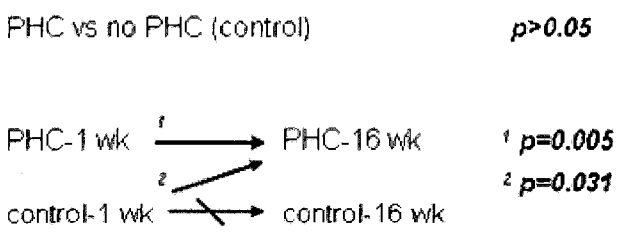

Figure 2.5: Pairwise comparisons of fungal community structure (genotypes) in PHC-treated and control systems at 1 and 16 weeks. Significant differences (MRPP) are represented by dark arrows and corresponding p-values; light (slashed) arrows represent no differences between groups.

Plant effects

Fungal community structure for different plant systems is represented along the first and third axes of the same NMS ordination (Figure 2.6). The clearest distinction is between single- and double-plant systems (represented by closed and open symbols, respectively). The single lingonberry (closed triangles) systems appear grouped towards the left side of the plot whereas single pine systems (closed squares) are more broadly distributed. Birch communities (closed circles) are distributed within the single pine and lingonberry clouds. Pairwise analysis using MRPP revealed that the pine and lingonberry communities from the double-plant systems differed significantly from communities from each of the single-plant systems, as well as from each other ($p<0.001$ in all cases). Single pine system communities varied significantly from both birch ($p=0.032$) and lingonberry ($p=0.028$) communities, which did not vary significantly from each other (note incomplete birch dataset).

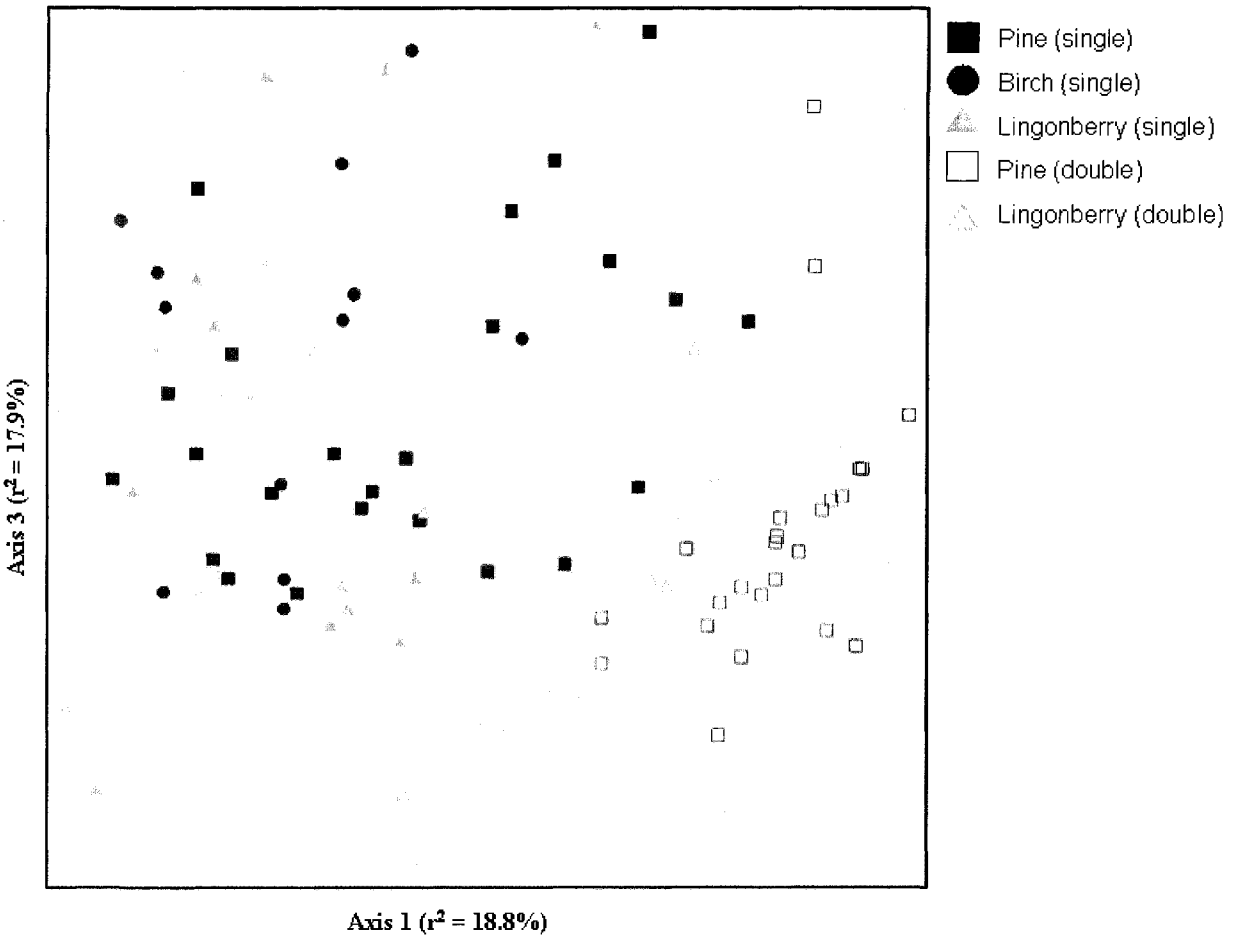

Figure 2.6: NMS ordination of mycorrhizal fungal communities of single (closed) and double (open) plant systems (stress = 19.66; instability = 0.12).

Separate analyses of pine and lingonberry communities reiterated differences between the single- and double-plant systems. In both cases, genotype richness was greater in the double-plant systems. For pine, single-plant systems had an average of 5.54 genotypes per root system (n=24) compared to an average of 6.88 genotypes per root system (n=24) in double-plant systems. For lingonberry, single-plant systems averaged 6.20 genotypes (n=25) and double-plant systems averaged 7.63 genotypes (n=24). No differences in genotype richness were associated with other treatment variables. For both plants, differences between fungal communities in single- compared to double-plant systems had p-values of <0.001.

Organic layer effects

To eliminate the double-plant effect on the analysis of soil layers, 24 FH, 19 CWD, and 18 FHoil fungal communities were compared between single-plant systems only. Of a total of 61 genotypes, 31 were found only in FH soils, while CWD and FHoil soils each had six unique genotypes. NMS analysis again gave a three-dimensional solution with a final stress value of 17.64 and instability of 0.024. The ordination shown in Figure 2.7 shows the broad distribution of genotypes in FH soils (dark squares), which reflects the heterogeneity of fungal communities found in these systems. Along the second and third axes, the FHoil soils (inverted triangles) are less broadly distributed than the FH soils; the CWD soils (open diamonds) form the tightest cluster in the center of the ordination. Fungal community structure varied significantly between all three types of soil systems: FH and CWD ($p=0.016$), FH and FHoil ($p=0.048$), and CWD and FHoil ($p=0.027$).

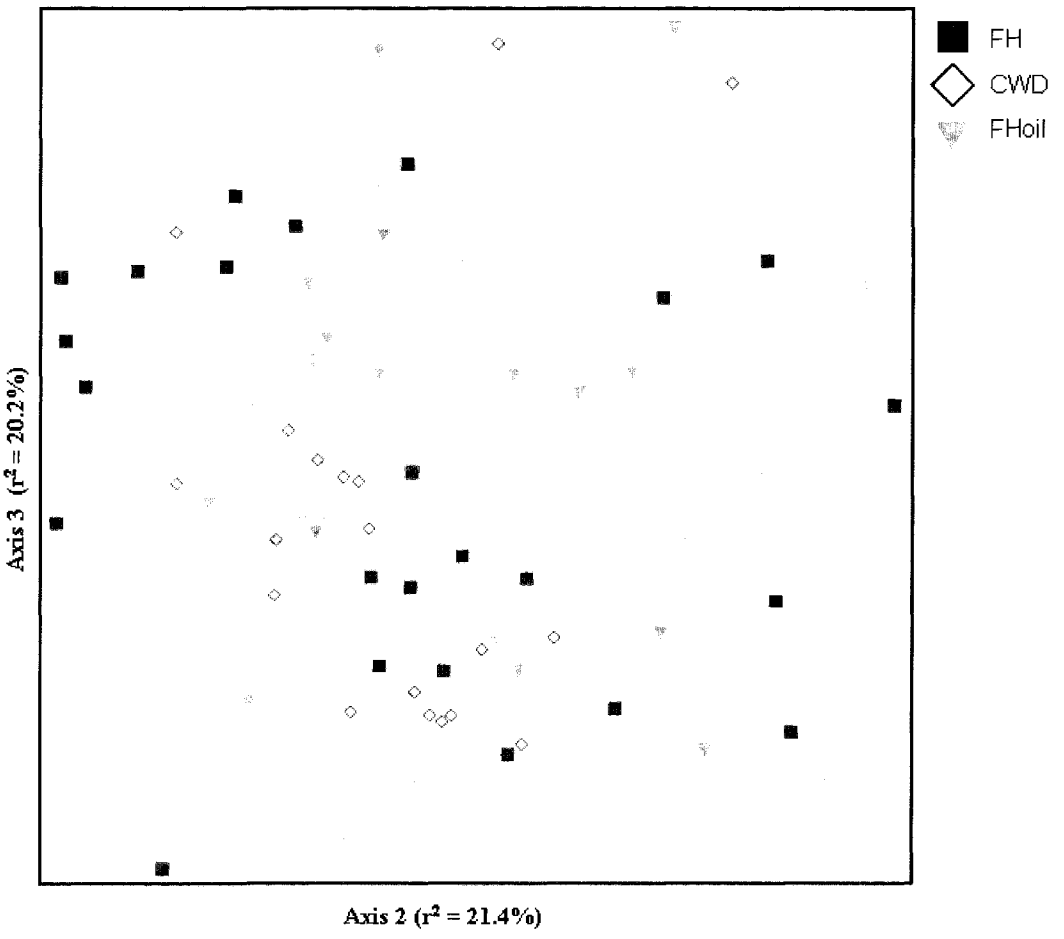

Figure 2.7: NMS ordination of organic soil layer (FH, CWD, and FHoil) effects in single plant systems (stress = 17.64; instability = 0.024)

MRPP comparisons between PHC treated and untreated (control) systems within soil groups showed that most differences were related to soils, but that some may have been related to PHC treatment. No PHC-treated soil systems (i.e. FH, CWD, or FHoil systems) varied significantly from each other; all differences were related to the control soils. Fungal communities in FH control soils varied significantly from both CWD ($p=0.024$) and FHoil ($p=0.041$) control soils, but CWD and FHoil communities did not vary significantly from each other. Fungal communities in FHoil control soils varied significantly ($p=0.016$) from PHC treated FH soils, but not from either PHC treated CWD or FHoil soil communities.

Differences between fungal communities in the different soil systems were only found within control groups (i.e. FH, CWD, and FHoil all varied significantly from one another), as well as between the control FHoil and treated FH and CWD (but not between treated and control FHoil soils). Lingonberry communities showed the same soil effects.

Laccase activity

Most ECM fungal root tips showed some level of laccase activity (i.e. development of a green color over 24 h), as shown in Figure 2.8. The greatest level of activity was associated with *Rhizopogon-Suillus* 1 and the two Russulaceae species that dominated pine roots (the Russulaceae ECM associated with birch was not tested). In the numerous trials conducted, Russulaceae (root tips) and *Rhizopogon-Suillus* 1 (root tips and/ or extraradical mycelia) harvested from pine seedlings from different soils and PHC treatments consistently produced a light green colour within the first hour of the assay, and a deep green colour by the 16-h mark. The *Rhizopogon-Suillus* 2 ECM did not often give positive results for laccase activity,

but sometimes produced a light green colour by 16 or 24 hours. *Amphinema* ECMs usually oxidized a low level of ABTS within the first hour of the assay; this light green colour did not generally deepen over the subsequent 23 hours, possibly indicating a low level of laccase secretion by this ECM. With respect to the ascomycetes, MRA usually showed low laccase activity within the first hour and substantial oxidation of ABTS by 16 and 24 hours. Laccase activity was seldom observed with *Cenococcum* ECMs; no enzyme activity was ever observed for E-strain. Neither soil type (i.e. organic layer) nor PHC treatment had any visible effect on laccase activity associated with these ECM root tips.

For ERM hair roots, little laccase activity was ever observed; however, in one trial (n=5), hair roots from PHC-treated soils showed a strong positive reaction whereas roots from untreated soils showed no activity.

	Colour Development		
	1 h	**16 h**	**24 h**
ECM			
Russulaceae 1	+	++	++
Russulaceae 2 (*Lactarius*)	- (+)	++	++
Rhizopogon-Suillus 1	++	++	++
Rhizopogon-Suillus 2	-	- (+)	- (+)
Amphinema	+ (-)	+	+
MRA	- (+)	+ (++)	+ (++)
Cenococcum	-	- (+)	- (+)
E-strain	-	-	-
ERM			
ERM-PHC	+ (-)	+ (-)	+ (-)
ERM-no PHC	-	-	-

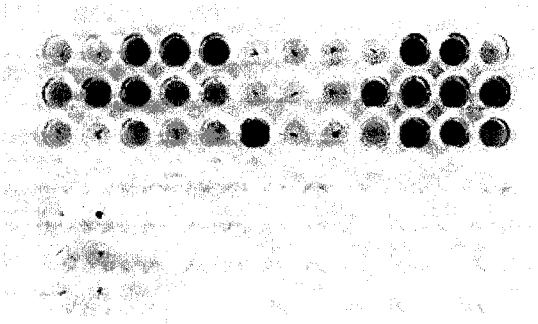

Figure 2.8: Table showing laccase activity of ECM morphotypes and ERM hair roots assessed by intensity of colour development (-, none; +, pale green; ++, dark green) at 1, 16 and 24 h incubation times with ABTS on the left. Symbols in brackets indicate less typical reactions over numerous trials (n=5+); Photograph showing range of colour development for ECMs (n=3, vertically) incubated in microplate wells with ABTS for 24+ h on the right.

Discussion

We found that PHCs, when applied to plant-soil systems at rates comparable to a large terrestrial oil spill event (i.e. equivalent to several tonnes of crude oil per hectare), had little impact on established or developing mycorrhizal fungal communities over 16 weeks of study.

PHC impacts on mycorrhizal communities

Detailed morphological analysis showed that the ECM morphotypes described here represent a sub-set of ECMs commonly reported from northern forest soils (Rosling *et al.*, 2003). These included *Cenococcum*, *MRA*, E-strain, *Amphinema*, three Russulaceae (including *Lactarius*), two *Rhizopogon-Suillus*, and two Thelephoraceae types, as well as three unidentified ECMs. Notably absent from our bioassay were *Piloderma* ECMs, even though their distinctive yellow mycelia were prevalent in FH, CWD, and particularly FHoil organic layers of the sub-boreal forest field site. *Piloderma* has been associated with greater N content that may be more typical of mature forest floors, which may explain why it was not associated with the seedling roots in the current study (Lilleskov *et al.*, 2002). It is thought that *Piloderma* may specialize in efficient N uptake or increase nutrient availability through enzymatic degradation of organic substrates, potentially including PHCs. Thus, preservation of organic layers (i.e. *Piloderma* habitat) following PHC contamination may be an important management strategy for sustaining this ecological function. *Cortinarius*, which also tends to be associated with a high C:N ratio in mature forest soils (Lindahl *et al.*, 2007) and may possess biodegradation potential, was also not observed in this study.

We found that most ECMs appeared unaffected by PHC treatment at any of the levels tested (i.e. ranging from approximately 7 to 22 tonnes ha^{-1}). In one of the few comparable studies of PHC-contaminated agricultural and forest soils (i.e. initiated following an oil well blowout in northern Italy that contaminated an area of 1,500 ha with 18,000 m^3 of crude oil), ECM fungal responses ranged from no negative effects to reduced biomass and colonization potential (Nicolotti and Egli, 1998). In subsequent experiments, in which different levels of PHCs were added to culture media, Nicolotti and Egli (1998) found that some fungal species were inhibited by oil whereas others (e.g. *Laccaria* sp.) appeared to grow better with PHCs, even at very high concentrations. Although the current study encompasses more of the complexity of the forest soil environment (particularly with respect to source of C via plant photosynthesis) and is not directly comparable to culture-based studies, a similar spectrum of ECM responses to PHC contaminants was observed. For example, *Cenococcum* ECMs may have been inhibited by PHCs. The dry, flaccid appearance of *Cenococcum* on some root systems may have partially resulted from toxicity of PHC chemicals, but was also likely due to physical changes in the soil habitat (i.e. change in water holding capacity of the FH layer) that occurred concurrently with PHC contamination. Soil drying may have been exacerbated in our model plant – soil systems that lacked a protective moss layer and forest canopy, and were thus potentially exposed to an even greater moisture loss due to evaporation compared to field conditions. The consequences of *Cenococcum* reduction as a functional component of the community are currently not known. On the other hand, rhizomorphic development of *Rhizopogon-Suillus* types may have been enhanced by PHCs, as hyphal fans proliferated into the contaminated mineral soil (biomass was not measured). The resulting expansion of mycorrhizosphere space would be expected to support diverse microbial communities

involved in the biodegradation of PHC substrates. No differences in distribution or abundance were observed for the Russulaceae types, which accounted for the majority of ECMs on all pine and birch root systems, regardless of PHC treatment.

The diversity (i.e. Simpson diversity) of ECM communities on pine and birch root systems generally decreased with greater PHC concentrations, but this trend was neither consistent nor usually significant. At any one of the six sampling times, there were no substantial changes of dominant ECMs in PHC treated systems compared to controls. ECM and ERM fungal community structure (based on genotypes) also did not vary between PHC treated and untreated (control) systems. As the plant – soil systems were treated with fairly high and ecologically relevant concentrations of crude oil, these results indicate that the intact organic soil layer, ECM mantle, and ERM hair root provided some level of protection against the potential toxicity of PHC constituents (Blakely *et al.*, 2002). As also reported elsewhere (Setälä *et al.,* 2000), our results suggest that maintenance of the ecological integrity of plant – soil systems provides resilience to environmental stress such as PHC contamination.

The temporal aspect of assessing mycorrhizal response to PHC treatment in dynamic systems was emphasized in this analysis. The morphological studies revealed a general increase in ECM richness and abundance over time, which was generally not altered by PHC treatment. As seedling root systems and mycorrhizal associations were still growing and developing at the time of PHC treatment, changes in diversity may have been due to increased ability to identify ECMs, or as a result of fungal succession on root tips, as has been reported from other studies (Massicotte *et al.*, 1999). The molecular studies also indicated shifts in ECM

fungal community structure over time and these time-related differences were only significant in the PHC treated systems. Thus, important questions with respect to ecosystem sustainability are whether PHC contamination alters the trajectory of mycorrhizal community structure in the long-term, and whether ecosystem health may be negatively impacted in the future as a result. Long term studies are required to address these questions.

ECM and ERM fungal community structure was largely determined by properties associated with host plants than by PHC contamination. Differences in community structure between single-plant systems reflect the many potential plant-fungal combinations that occur in repeatable units across landscapes (Allen *et al.*, 2003). More information on the individual physiologies of these symbioses is required to understand their roles in ecological processes and to predict how communities may adapt to future disturbances. Although fungal community structure varied between all host plant systems, differences were most striking between the single- and double-plant systems (i.e. more complex systems). These differences appeared to be due to greater genotype richness per root system, as well as altered genotype abundance patterns in the double-plant systems. Even though pine and lingonberry mycorrhizas shared the same rhizosphere in the double-plant systems, the ECM and ERM fungal communities remained distinct. The mycorrhizal community patterns observed in double-plant systems may represent the non-linear, emergent properties associated with complex systems whereby the influences of mycorrhizal fungi on plant populations and communities are not merely the sum of effects on the individuals within populations (Dahlberg, 2001; Allen *et al.*, 2003).

In addition to assessing the integrity of established mycorrhizal fungal systems, we also tested the ability of seedlings to germinate and grow in contaminated soils (i.e. FHoil systems), which is an important consideration in the context of environmental restoration. In these chemically complex systems, sensitivity to the initial conditions may create limits to mycorrhizal diversity (Allen *et al.*, 2003), although this was not evident in either of our morphological or molecular studies. Nicolotti and Egli (1998) found that crude oil (50 ppm hand-mixed into homogenized forest soil) did not inhibit Norway spruce or poplar seed germination or kill seedlings. In our study, using a much greater PHC concentration, longer weathering period, and reconstructed soil layers, we also found that germination of pine seeds did not appear to be inhibited by the presence of weathered crude oil. Furthermore, seedling growth/ mycorrhizal colonization was sometimes more vigorous in the previously contaminated soils compared to pristine (FH and CWD) layers. Birch seeds appeared to have had lower rates of germination, possibly due to interference of PHC chemicals with initial interactions between germinating roots, fungal propagules, and mycorrhizal helper bacteria (Garbaye, 1994). However, the seedlings that grew in FHoil layer showed no apparent differences to seedlings germinated in the other organic soil layers (i.e. FH and CWD layers). The survival of lingonberry seedlings also did not differ between FH, CWD, and FHoil soil systems.

Potential for direct mycorrhizal role in biodegradation

Nicolotti and Egli (1998) reported that some ECM fungi surviving in contaminated forest soil may metabolize chemicals in crude oil. The distribution of laccase-secreting fungi represents a part of the oxidative potential in soils (Luis *et al.*, 2005). We found that the metabolic

potential for laccase-mediated PHC biodegradation appears to exist among the dominant members of ECM fungal communities including two Russulaceae (one *Lactarius*), *Rhizopogon-Suillus* 1, and *Amphinema* types. High extracellular enzyme activities have been reported for *Lactarius* and *Russula* species grown in culture (Gramss *et al.*, 1998) and laccase-like genes have been amplified from *Lactarius*, *Russula*, *Piloderma* and *Tylospora* fungi and soils (Chen *et al.* 2003; Luis *et al.*, 2005). Increased extracellular enzyme activity and expression of genes coding these enzymes have been found at the hyphal fronts of ECM systems advancing in the humus of microcosms (Timonen and Sen, 1998; Donnelly and Entry, 1999). The function of fungal laccase in the carbon cycle in soils, especially in the formation, stabilization and degradation of the organic matter, is also of ecological interest (Luis *et al.* 2004).

From our study, we know that the Russulaceae types dominated pine root systems in all soil systems, both PHC treated and untreated, and could potentially influence large volumes of forest soils (if distribution patterns are consistent with our bioassay). The *Rhizopogon-Suillus* 1 ECM, also influenced large volumes of mineral soil via rhizomorphic proliferation, which may have even have been enhanced by high levels of PHC contamination. *Rhizopogon-Suillus* ECMs are not well studied, partly because they are often found deeper in the soil profile than most researchers sample (Rosling *et al.*, 2003). *Piloderma* ECMs were not described from our study, but it is likely that laccase-secreting *Piloderma* ECMs are abundant in mature forest soils. These results indicate that soils with intact ECM systems may possess high biodegradative potential for PHC contaminants via the combined activities (syntrophic metabolism) of microorganisms in the mycorrhizosphere.

We did not find evidence of enhanced laccase activity (i.e. intensity of laccase reactions) resulting from exposure of pine ECMs to PHC substrates, as has been reported from studies in which a variety of different aromatic compounds were tested (Burke and Cairney, 2002). It could be that the ECMs in this study were directly exposed to lower PAH concentrations in the crude oil-contaminated soil compared to other studies. Alternatively, ECM fungi inhabiting soils influenced by organic layers rich in lignin and humus may exist in a state of enhanced laccase expression and activity. The warm, wet greenhouse conditions may have further enhanced laccase activity through provision of favourable decomposition/ biodegradation conditions.

We generally did not observe much evidence of laccase activity when hair roots of lingonberry were tested, even though ERM fungi (e.g. *R. ericae*) have been reported to have well-developed saprotrophic abilities (Burke and Cairney, 2002; Perotto *et al.* 2002). In one set of trials, laccase activity was consistently observed with hair roots that were recently exposed to PHCs, but not with control roots. This result was not repeated, so no conclusions can be drawn from this. As very little is known regarding biodegradation capacity in ERM systems, further investigation is warranted.

Conclusions

In this study, we found that established ECM and ERM systems appear resilient to toxic effects of crude oil, which was related to the integrity of the plant – soil systems. In addition, ECM systems may directly enhance PHC biodegradation at the fungal – soil interface

through secretion of laccase, as well as indirectly aiding biodegradation through provision of colonization surfaces and co-metabolic substrates for associated bacterial communities. Thus, mycorrhizal systems may also be part of the remediation solution for PHC contaminated forest sites within a sustainable ecosystem management context.

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Chapter 3: Enhanced biodegradation of petroleum hydrocarbons in the mycorrhizosphere of sub-boreal forest soils

Abstract

The dynamics of petroleum hydrocarbon (PHC) biodegradation in boreal forest soils are not well understood. We used a bioassay approach to determine whether differences in mycorrhizosphere-associated bacterial communities corresponded to differences in PHC biodegradation patterns. Surface-sterilized seeds (*Pinus*, *Betula*, *Alnus* sp.) or seedlings (*Vaccinium* sp.) were planted into Conetainer^{TM} pots containing reconstructed soils: an organic layer (mor humus, coarse woody debris, or previously oil-contaminated humus) overlying sandy mineral horizons (Ae and Bf) of field-collected forest soils obtained from central BC, Canada. After 4 months, BC light crude oil (219 mg cm^{-2}) was applied to the soil surface around the seedling stem; systems were destructively sampled at 1 and 16 weeks following treatment. Concentrations of PHCs in 4 fractions (based on equivalent normal straight-chain boiling point ranges) were determined using acetone-hexane extraction followed by GC-FID analysis. Genotypic profiles of root-associated bacterial communities were generated using length heterogeneity-PCR analysis of 16S rDNA; metabolic profiles were based on C substrate use after 7 weeks. Nearly all plant-organic soil layer combinations showed significant loss of nC10-nC16 fraction PHCs from 1 to 16 weeks, indicating an inherent capacity for biodegradation within these soils. Total PHC (nC10-nC50) concentrations declined significantly in only planted (pine-woody debris and birch-humus) systems, reinforcing the importance of the (mycor)rhizosphere for supporting degradative microbial communities. Multivariate analyses showed that (mycor)rhizosphere type and complexity influenced bacterial community structure, but that this was not related to PHC

biodegradation. The level of PHC contamination used in this study appeared to have minimal impact on soil bacterial community structure or broad metabolic functions.

Introduction

Boreal ecosystems are increasingly exposed to petroleum hydrocarbon (PHC) contamination due to expanding natural resource extraction activities (Kanaly and Harayama, 2000). Long-term studies of oil-contaminated forest soils have reported reductions in PHC levels due to biodegradation (or biotransformation) by indigenous microbial communities (Braddock *et al.,* 2003; Prince *et al.,* 2003). From culture and microcosm experiments, many ubiquitous soil fungi and bacteria have been demonstrated to biodegrade numerous PHC compounds (Heinonsalo *et al.,* 2000; Sarand *et al.,* 2000; Genney *et al.,* 2004; Corgié *et al.,* 2003) and some genetic and biochemical pathways have been elucidated (Meharg and Cairney, 2000; Burke and Cairney, 2002; Watanabe, 2002; Díaz, 2004). It is generally accepted that the capacity to biodegrade PHCs is intrinsic in most soils (Meharg and Cairney, 2000; Chaillan *et al.,* 2004; Delille *et al.,* 2004) and requires metabolic synergy among different functional guilds of organisms, including mycorrhizal fungi and the bacterial communities closely associated with the mycorrhizosphere (Burke and Cairney, 2002; Díaz, 2004; Chaudhry *et al.,* 2005). Very little is currently known of the dynamics of PHC biodegradation as it occurs within the mycorrhizosphere of forest soil systems (Robertson *et al.*, 2007).

Soil microbial communities may shift in response to changes in environmental conditions (Watanabe, 2002), but exhibit high resilience to environmental stresses when soil organic layers (e.g. humus, woody debris, etc.) are not severely disrupted (Setälä *et al.,* 2000). Major impacts of PHCs on forest soil microbial communities appear to be associated with disturbances to water, nutrient and oxygen regimes related to the hydrophobicity and fluidity of oily products (Tarradellas and Bitton, 1997). Spilled PHCs initially spread laterally within

the lignin-rich humus of the forest floor; eventually, lighter fractions move down the soil profile, along the paths of roots and fissures (Trofimov and Rozanova, 2003; Suleimanov *et al.,* 2005), where changes with depth in soil chemical and mineralogical properties create contrasting habitats for microorganisms (Dickie *et al.,* 2002; Rosling *et al.,* 2003). PHC contamination is expected to lead to an initial loss of bacterial diversity, followed by rapid proliferation of metabolically competent populations capable of inhabiting the new environmental conditions imposed by the chemical contaminants (Gramss *et al.,* 1998; Díaz, 2004). Part of this rapid adaptation within microbial communities results from lateral transfer of mobile genetic elements carrying genes for PHC biodegradation (Sarand *et al.,* 2000; Díaz, 2004) and is controlled by the composition and functional redundancy of the community originally present (Setälä *et al.,* 2000; Delille *et al.,* 2003). Changes in bacterial community structure attributed to PHC contamination have been observed for several years after the initial spill event occurred (Lindstrom *et al.,* 1999).

Northern forests are dominated by plants forming ecto- (ECM) and ericoid (ERM) mycorrhizal symbioses with fungi expected to have well-developed saprotrophic activities (Read and Perez-Moreno, 2003). Mycorrhizal establishment alters the quality and quantity of root exudates that supply energy to the large chemo-organotrophic biomass associated with the mycorrhizosphere (Rygiewicz and Anderson, 1994; Nannipieri *et al.,* 2003; Morgan *et al.,* 2005). Interactions between mycorrhizal fungal mycelia and associated bacterial communities are important mechanisms for accessing mineral nutrients from organic substrates (Burke and Cairney, 2002; Sen, 2003) and also seem crucial for cometabolic biodegradation of PHCs in contaminated soils (Sarand *et al.,* 1998; 2000). Mycorrhizosphere

development and function play central roles in controlling bacterial communities and their biodegradation activities in lignin-rich humus and PHC-contaminated soils (Heinonsalo *et al.,* 2000). Enhanced PHC biodegradation in mycorrhizosphere soils (i.e. mycorrhizosphere effect) is attributed to the greater metabolic activities of higher densities of microorganisms (Heinonsalo *et al.,* 2000; Corgié *et al.,* 2003; Siciliano and Germida., 1998).

Various combinations of host plants and mycorrhizal fungi may create specific mycorrhizosphere characteristics that are important with respect to biodegradation capacity (Rygiewicz and Anderson, 1994; Perez-Moreno and Read, 2000; Selmants *et al.,* 2005). For example, plants that also form symbioses with N-fixing organisms (e.g. *Alnus* sp. with *Frankia*) have increased availability of N as well as C in the mycorrhizosphere (Selmants *et al.,* 2005; Roy *et al.,* 2007). Interactions between overstory (predominantly ECM plants) and understory (plants forming ERM or arbuscular mycorrhizas) vegetation may also alter capacity for biodegradation (Read and Perez-Moreno, 2003). Thus, host-fungal effects on C and nutrient availability should not be ignored in biodegradation capacity studies (Corgié *et al.,* 2003).

In the current study, we examined relationships between PHC biodegradation and indigenous microbial communities in reconstructed sub-boreal forest soils. We compared biodegradation patterns in different plant-soil systems that varied with respect to organic soil layer (i.e. forest floor, coarse woody debris, or forest floor previously contaminated with PHCs) and plant (*Pinus*, *Betula*, *Alnus*, or *Vaccinium* sp.) characteristics. In these systems, organic soil layers provided the initial inoculum for the developing rhizospheres; the

functional composition of these communities was expected to be influenced by the relative abundance of lignin and/ or previous exposure to PHCs. Microbial community structure and function were expected to be further influenced by individual (mycor)rhizosphere properties. For example, although pine and birch both support ECM communities, they differ with respect to density and depth of fine root tips. Alder forms symbioses with both ECM and N-fixing communities, while lingonberry forms ERMs. Differences between single- and double-plant (i.e. pine and lingonberry) systems were also assessed, as well as effects on bacterial communities by the PHC treatment itself. Ultimately, the goal of this study was to determine whether differences in bacterial community (genotypic and metabolic) profiles corresponded to changes in biodegradation patterns in an ecologically relevant context.

Materials and methods

Field site

The Kenneth Creek field site is located in the wet, cool subzone of the sub-boreal spruce (SBSwk1) biogeoclimatic zone of central British Columbia, Canada, about 100 km east of Prince George (53°34'N, 122°47'W). In 1982, the forest was logged and burned, then subsequently planted with lodgepole pine (*Pinus contorta* Dougl. Ex Loud. var. *latifolia* Engelm.); currently, the site is a mature, even-aged pine stand with small hybrid white spruce (*Picea glauca* x *engelmannii* Parry *ex* Engelm.) and lesser numbers of western hemlock (*Tsuga heterophylla* (Raf.) Sarg.). Young subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.) and sitka alder (*Alnus crispa* var. *sinuata* (Reg.) Rydb.) are present at the edge of the forest, along the access road; western redcedar (*Thuja plicata* Donn) and trembling aspen (*Populus tremuloides* Michx.) are also present in an unlogged stand across the main road. The site has

a thick understory of oval-leaved blueberry (*Vaccinium ovalifolium* Sm.); mosses and lichens (e.g. *Peltigera*) cover the forest floor, with some *Lycopodium* species.

The soils on this site were described by Arocena and Sanborn (1999). Soils are classified as Eluviated Dystric Brunisols (Soil Classification Working Group, 1998), and consist of sandy parent material with low clay content and few coarse fragments. The forest floor is mor humus, from 2-5 cm thick with copious fungal mycelia present. The C:N ratio of the forest floor is approximately 50 and the pH (water) is ~ 4.2. Gray Ae horizons are generally 1-2 cm thick, with thicker pockets in some areas. Red Bf horizons extend to almost 30 cm, beneath which are Bm (27-60 cm), BC (60-100) and C (> 1 m) horizons. The C:N ratios of the Ae and Bf horizons are about 20 and 13, respectively. The pH (water) of the Ae horizon is 4.2 while the pH of the Bf horizon is about 4.8, increasing with depth to about 6.0 at the transition to the C horizon. Fine roots are found at depths greater than 1 m. Large coarse woody debris (i.e. downed trees), the legacy of past forest management, is abundant all over the site.

Organic layers and the top 20-30 cm of mineral soils (Ae and Bf horizons) were collected from the forest site in September of 2005 and 2006. The organic layers included the mor humus forest floor (FH) that had been undisturbed for approximately 20 years, coarse woody debris (CWD) in an advanced state of decay (i.e. decay class 5), and previously PHC-contaminated mor humus (FHoil) that had weathered *in situ* for four months throughout the summer. The FHoil soils were collected in the first year only. In May of 2005, 2.0 L of BC light crude oil (Husky Refinery, Prince George, BC) was applied to each of three 1-m^2 plots

with the moss layer (but not seedlings and other plants) removed. The oil had been previously bubbled with N_2 gas to remove the light and volatile compounds; a watering can was used for even application in the field. All soils were stored at 4°C prior to use in bioassay experiments, which commenced within 10 days of soil collection.

Bioassay and PHC treatment

Forest soils (i.e. Ae [~1 cm] and Bf [~15 cm] mineral soil layers beneath organic [FH, CWD, or FHoil] soil layers [~2 cm]) were reconstructed in Cone-tainer™ pots (3.8 x 21 cm, Stuewe and Sons, Corvallis, Oregon) with two clay pellets in the bottom to prevent soil loss (Setälä *et al.,* 2000). Mineral soils (Ae and Bf layers) were homogenized and sieved through (1 cm^2) screens prior to potting. Surface-sterilized seeds of lodgepole pine (*Pinus contorta* var. *latifolia*), white birch (*Betula papyrifera* Marsh.), and sitka alder (*Alnus crispa* var. *sinuata*), collected from the SBS and obtained from the Ministry Tree Seed Center, (Surrey, BC (Seed lots DWD20050009A (location 079-B-008), DWD20050009B (location 094-E-015), and DWD20050047A (location 002-E-001), respectively), were planted into each pot. Lingonberry (*Vaccinium vitis-idaea* L.) seedlings were obtained from Birch Creek Nursery (Prince George, BC) and planted into 10x10x10 cm pots (i.e. Ae [~1 cm] and Bf [~7 cm] mineral soil layers beneath organic [~2 cm] soil layers). All pots were placed in the greenhouse (22°C day temperature, 15°C night temperature, and 16 h photoperiod) and fertilized once a month (5 mL of NPK fertilizer; providing 100 ppm each of NPK) for the first four months following seeding/ planting. The plants were watered two or three times per week for the duration of the experiment. Single-plant (pine, birch, alder, and

lingonberry) systems were established in 2005-06; double-plant (pine and lingonberry) systems were established in 2006-07.

Seedlings and mycorrhizas were well established after four months of growth. At this time, 3 mL BC light crude oil (with volatiles removed as for the field application) was pipetted onto the organic soil surface of each pot, around, but not touching, the seedling stem. PHC concentration (219 mg cm^{-2}) corresponded to an application rate of 21,900 kg ha^{-1} (i.e. ~22 tonnes ha^{-1}). The smell of crude oil dissipated in the greenhouse pod within the first week following PHC treatment and no PHC loss was observed from the bottom of the pot (i.e. no sheen on the wet surface below) after watering for the duration of the experiment. These observations provided some confidence that observed PHC losses between 1 and 16 weeks were primarily due to biodegradation.

Experimental design and sampling

Experiments followed a completely randomized block design with a planned full factorial structure for plant, organic soil layer and PHC treatments (Table 3.1).

Table 3.1: Summary of plant, organic soil layer, and PHC treatment variables for 2005-2006 (single-plant) and 2006-2007 (double-plant) studies (n=3)*.

Plant	Organic Soil Layer	PHC
Pine [P]	Forest floor [FH]	No PHC (control)
Birch [B]	Coarse woody debris [CWD]	PHC (219 mg cm^{-2})
Alder [A]	Contaminated forest floor [FHoil]	
Lingonberry [L]	No organic soil [NO] (control)	
Pine + Lingonberry [P+L]		

* n<3 (due to poor germination) for birch –CWD, birch –FHoil, and all alder treatments; these treatments were removed from PHC and DNA analyses.

For soil, PHC, and DNA analyses, all three soil layers (organic, Ae, Bf) of PHC-treated and untreated control soil systems (n=3) were destructively sampled at 1 and 16 weeks following PHC treatment. Treatments included single-plant (pine [P], birch [B], lingonberry [L], and a control [N] with no plant (i.e. no mycorrhizosphere)) and organic soil layer (mor humus [FH], coarse woody debris [CWD], PHC-contaminated mor humus [FHoil], and a control [NO] with no organic layer) combinations; double-plant (P+L) systems and unplanted controls (N) were grown in FH and CWD organic soil layers. Individual pots were emptied into trays; plants were gently removed (for future mycorrhizal community analysis) from the soil with as little disturbance as possible to the reconstructed soil horizons. Approximately 1 g (wet weight) of each soil layer was collected in duplicate in 20 mL pre-weighed glass vials (Fisher Scientific, Ottawa, Ontario). One set of samples was dried overnight in a 105°C oven to obtain soil dry weights (i.e. soil moisture contents); the other set was stored at 4°C until PHC extraction. The remaining soils were combined within treatment groups and then collected and air-dried for soil nutrient analysis and pH. Root systems (intact, but with the shoot excised) were shaken free from the soil and then washed in 35 mL sterile H_2O. Roots were then divided into 2 mL tubes and stored at -20°C until DNA extraction.

For bacterial community level physiological profile (CLPP) analysis, 60 plant – organic soil systems (n=2) were harvested seven weeks after PHC treatment in 2006. Treatments included PHC-treated and control pots for all single plant (pine [P], birch [B], alder [A], lingonberry [L], and no plant [N]) and organic soil layer (FH, CWD, FHoil) combinations. After initially shaking the roots to remove excess soil, the intact root systems were placed in 50 mL tubes containing 35 mL of sterile dH_2O. Deionized water was used instead of buffer

as it does not contain nutrients that could potentially interfere with extracellular enzyme analysis and because buffering may alter enzyme activity or bioavailability of contaminants/ substrates (Palmroth *et al.,* 2005). The tubes were gently shaken for 20 s to remove mycorrhizosphere soil clinging to the roots. This solution was used to inoculate Biolog EcoPlates™ (Biolog, Hayward, CA).

PHC extraction and quantification using GC-FID

A sequential shake method was used for PHC extraction (Schwab *et al.,* 1999; Siddique *et al.,* 2006). Ten millilitres of acetone:hexane (1:1, vol/vol) were added to approximately 1 g of soil (wet weight) in 20 mL glass vials. The vials were shaken on a reciprocating platform shaker at 120 cycles per minute for 30 min. Soil particles were allowed to settle before the extract was removed. This process was repeated three times and extracts were then combined.

The extracts were cleaned using a silica gel column procedure to remove polar organic compounds (CCME, 2001). Glass columns (inside diameter of 16 mm) were plugged with glass wool and filled with approximately 60 mm of 70–230 mesh Grade 60 Å activated (heated to 110°C for 12 h) silica gel followed by approximately 25 mm of ASC anhydrous sodium sulfate (dried at 400°C for 4 h). Approximately 10 mL of acetone:hexane (1:1, vol/vol) was used to condition the column prior to adding the 30 mL of PHC extract solution, which was accomplished by pipetting the solution into the top of the column and letting it run down the glass to the top of the sodium sulfate layer. After the extract had passed through the column, the column was flushed with 10 mL cyclohexane to ensure all compounds of

interest were collected. The collection vessels were 40 mL glass vials with Teflon-lined caps. Two milliliters of toluene were added to each vial and the caps were left open to evaporate the lighter organic solvents. Once most of the solvents had evaporated, the concentrated PHC extracts were transferred to 2 mL GC tubes for the final evaporation, and topped up to exactly 2 mL with toluene if necessary. The PHC extracts were stored at 4°C until GC-FID analysis. Acetone, hexane (GC grade) and other chemicals (AR grade) were purchased from Fisher Scientific, Ottawa, Ontario.

The PHC extracts were analyzed on a Varian Model CP 3800 Gas Chromatograph (GC) equipped with a flame ionization detector (FID). A 15 m x 0.25 mm ID with 0.25 μm film thickness ZB-5 capillary column (Phenomenex Torrance, CA) was used for the separation of the PHC extracts. Typically 1 μL of PHC extract was injected into the GC system using a Varian CP 8400 auto-sampler. Splitless injection mode was performed on the 1079 PTV injector and after 0.7 min, the split mode was activated at split ratio 10:1. Both the injector and the detector (FID) temperatures were kept at 320°C during the analysis. The capillary column temperature was initially held at 50°C for 1 min, then increased at 15.0°C min^{-1} to 110°C and further increased at 10.0°C min^{-1} to 300°C and held at 300°C for 10 min. The total run time was 34 min for each sample. The carrier gas (helium) was maintained at a constant flow rate of 1.5 mL min^{-1} for the whole analysis and no pressure pulse was used for the injection.

PHCs from crude oil were quantified using the CCME reference method for determining PHC fractions (each fraction based on equivalent normal straight-chain hydrocarbon (nC)

boiling point ranges) in soil (CCME, 2001). The PHC fractions F2 (nC10- nC16), F3 (nC16-nC34) and F4 (nC34-nC50) were determined according to the CCME Canada-Wide Standard for Petroleum Hydrocarbons in Soil – Tier 1 Method (CCME, 2001). Earlier work showed that the F2 fraction contained mainly aliphatics such as alkanes. The F3 fraction includes aliphatic hydrocarbons as well as PAHs such as fluorene ($C_{12}H_{10}$), phenanthrene ($C_{14}H_{10}$) and pyrene ($C_{16}H_{10}$); here, we divided the F3 fraction into F3a (nC16-nC23) and F3b (nC23-nC34) fractions. Previous studies have shown the F1 fraction (nC6-nC10) to be negligible and therefore it was ignored in this study. Peak retention times (i.e. peak maximums) of the external standards decane (nC10), hexadecane (nC16), nonadecane (nC19), eicosane (nC20), tricosane (nC23), dotriacontane (nC32), and tetratriacontane (nC34) (Sigma Aldrich, Oakville, Ontario) dissolved in toluene were used to determine F2, F3a, F3b and F4 regions on the GC-FID chromatograms (Figure 3.1); external standards were run concurrently with samples at concentrations of 10, 25, 50, 125 and 250 ppm.

PHC concentrations ($\mu g\ mL^{-1}$) were calculated by dividing the areas under the GC-FID curves by the response factor for the F2, F3a, F3b, F4 and total PHC (tPHCs). These values were then multiplied by the volume of the GC vial (2.0 mL) divided by the equivalent dry weight (g) of each soil sample to obtain concentrations in $\mu g\ g^{-1}$ (ppm). The final concentrations of PHCs were compared at 1 and 16 weeks by 1-way ANOVA ($\alpha = 0.05$) using Statistica 6.1 (StatSoft Inc., USA).

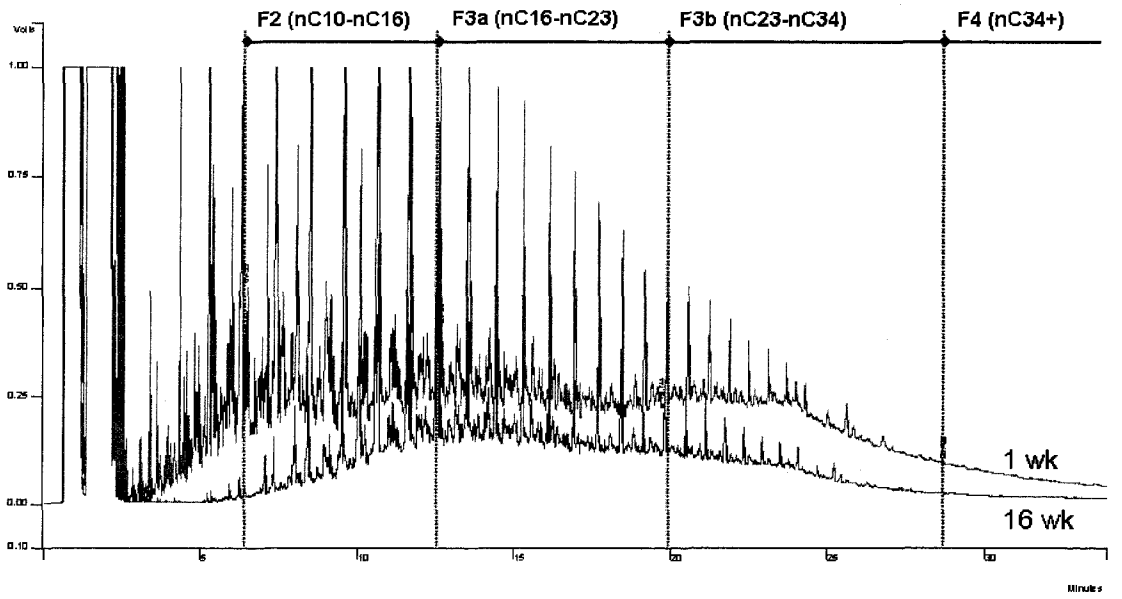

Figure 3.1: Overlay of two chromatograms generated from GC-FID analysis showing a reduction in PHC peak areas from 1 to 16 weeks. The vertical lines represent the boundaries (based on retention times of standards) for analysis of the F2 (nC10-nC16), F3a (nC16-nC23), F3b (nC23-nC34) and F4 (nC34-nC50) PHC fractions.

Soil nutrient analysis and pH

Organic (FH, CWD and FHoil) and mineral soil (Ae and Bf) samples from single-plant systems were analyzed for total C and N content using <100-mesh samples (air-dried, then ground in a Model MM200 ball mill; Retsch, Haan, Germany) by dry combustion using a Model 1500 NC Elemental Analyzer (Fisons, Milan, Italy). The pH of organic soil layers was measured in a 1:4 soil to deionized H_2O suspension while 1:2 suspensions (in deionized water) were used for mineral soils (Kalra and Maynard, 1991).

LH-PCR and fragment analysis

Mycorrhizosphere-associated bacterial communities (genotype richness and abundance) were characterized by amplicon length heterogeneity PCR (LH-PCR) using the D4 fluorescent

dye-labeled forward primer 27F (5'AGAGTTTGATCMTGGCTCAG) and unlabelled reverse primer 355R (5'GTCGCCTCCCGTAGGAGT) as described by Mills *et al.* (2003). Root systems were crushed in liquid nitrogen and DNA was extracted using a CTAB (hexadecyl trimethyl ammonium bromide) protocol with an extra phenol/chloroform-isoamyl alcohol (1:1) purification step (Fujimura *et al.,* 2008). DNA extracts were cleaned using the Wizard® PCR Preps DNA Purification System kit (Promega); cleaned extracts were resuspended in TE buffer. PCR reactions consisted of 3 μL DNA (diluted 1:50), 10X PCR buffer, 2 mM dNTPs, 50 μM $MgCl_2$, 10 μM forward and reverse primers (Proligo, CO), 0.7 U Platinum Taq DNA polymerase (Invitrogen Life Technologies), and nuclease-free water (Integrated DNA Technologies, Inc.) to a final volume of 30 μL. The DNA Engine DYAD™ thermocycler (MJ Research, Inc., Watertown, MA) conditions were as follows: initial denaturation for 1 min at 94°C, 35 cycles of denaturation (94°C for 45 s), annealing (52°C for 45 s) and extension (72°C for 1 min 30 s), and final extension at 72°C for 10 min. PCR products were run on 1.2% agarose gels to confirm amplification.

PCR products (2 μL) were loaded into a CEQ™ 8000 sequencer (Beckman-Coulter Inc.) along with CEQ 400 size standard mixture. Run conditions were 60°C separation temperature, 4 kV voltage, and 120 min separation time. Analysis was performed using the amplicon fragment length polymorphism (AFLP) program of the CEQ™ 8000 sequencer and the cubic model for size standard with a bin width of 1.5 bp. Peaks less than 11% of the total sample peak height were not included. Profiles from separate DNA extractions and PCR reactions were compared to assess reproducibility and suitability for analysis.

Relative abundance of genotypes was calculated by relativizing the fluorescent signal strength of each fragment peak to the total peak area within each sample (Osborne *et al.,* 2006). Community structure was assessed graphically with Nonmetric Multidimensional Scaling (NMS) using PC-ORD 5.0 software (McCune and Mefford, 1999; Mills *et al.,* 2006; Ramette, 2007). NMS was calculated on the basis of a Sørensen distance measure with 50 runs with real and randomized data and a maximum of 500 iterations to assess stability (instability criterion was 0.00001). A stepwise reduction in dimensionality (6D-1D) was used to minimize stress along with a random starting configuration (user-provided seeds). When possible (i.e. for balanced analyses), multivariate differences were tested statistically with nested permutational multivariate ANOVA (NPMANOVA) (Anderson, 2001); otherwise, univariate differences were tested with Multi-Response Permutation Procedures (MRPP) (McCune and Grace, 2002) to examine effects between treatments.

Community level physiological profiles

The metabolic potential of mycorrhizosphere bacteria was assessed by community level physiological profiles (CLPP) based on broad differences in C-substrate use patterns (Hofman *et al.,* 2004; Palmroth *et al.,* 2005). Mycorrhizosphere soil solutions (100 μL) were inoculated into Biolog EcoPlates™ (Biolog, Hayward, California) containing triplicate wells of 31 substrates commonly found in the rhizosphere (at least nine are considered to be constituents of plant root exudates) along with a redox dye (tetrazolium violet) that is reduced to a purple color during substrate oxidation. The plates were incubated in the dark at ~20°C. Reaction patterns (optical density at absorbance of 590 nm) were analyzed with a

microplate reader (Biolog Microstation plate reader and Biolog MicroLog 3 4.01A software) at 24-h intervals for five days.

For each plate, optical densities (OD_{590}) of triplicate wells were averaged and values for the water control subtracted from each averaged well value; negative values were adjusted to zero. Average OD_{590} values were calculated for substrate guilds: including amino acid (L-arginine, L-asparagine, L-phenylalanine, L-serine, L-threonine, glycyl-L-glutamic acid), amide/ amine (phenylethylamine, putrescine), carbohydrate (D-cellobiose, α-D-lactose, β-methyl-D-glucoside, D-xylose, I-erythritol, D-mannitol, N-acetyl-D-glucosamine), carboxylic acid (D-glucosaminic acid, D-galactonic acid, γ-lactone, D-galacturonic acid, 2-hydroxy benzoic acid, 4-hydroxy benzoic acid, γ-hydroxybutyric acid, itaconic acid, α-ketobutyric acid, D-mallic acid), polymer (tween 40, tween 80, α-cyclodextrin, glycogen), and miscellaneous (pyruvic acid methyl ester, glucose-1-phosphate, D,L-α-glycerol-phosphate) C substrates (Preston-Mafham *et al.,* 2002). Kinetic analysis of OD_{590} values by substrate guild was performed by calculating the area under each substrate use curve from 24 to 120 hours, which corresponded to the log (linear) phase of bacterial growth.

Area-under-curve values for PHC-treated and untreated communities were compared by 1-way ANOVA ($\alpha = 0.05$) with either plant or organic soil treatments pooled. Principal components analysis (PCA) was used to assess treatment and substrate effects on variation in bacterial community profiles. All analyses were conducted using Statistica 6.1 (StatSoft Inc., USA).

Results

PHC quantification from soils

Not all plant – organic soil combinations germinated in sufficient quantities to allow for a full factorial treatment with an equal number of replicates; thus, treatments with $n<3$ were not included in PHC analyses. For the single-plant systems (2005-06), triplicate samples were obtained at the two harvest dates (t = 1, 16 weeks) for P-FH, P-CWD, P-FHoil, B-FH, L-FH, L-CWD, L-FHoil and the unplanted controls (N-FH, N-CWD, N-FHoil), for a total of 30 pots (each with three soil horizons) per harvest. For the double-plant systems (2006-07), triplicate samples were obtained at the two harvest times for PL-FH, PL-CWD and the unplanted controls (N-FH, N-CWD), for a total of 12 pots per harvest.

Total PHC concentrations (sum of F2, F3a, F3b, F4 fractions) in organic layers of PHC-treated soil systems averaged over 150,000 ppm at 1 week (Figure 3.2). The high ppm was due, in part, to the lower particle density in organic compared to mineral layers. PHCs extracted from organic (FH, CWD and FHoil) soil layers accounted for 90% of the total PHCs extracted from each soil system; PHC levels in Ae layers were typically greater (~7.3%) than in Bf layers (~1%). In organic layers, PHC concentrations generally decreased over 16 weeks, with significant losses in FH ($p<0.001$) and CWD ($p=0.02$) soils only. The levels of PHC-like chemicals extracted from untreated (control) FH and CWD layers were very low, indicating that the GC-FID chromatograms largely represented the PHCs added to these systems (and not other soil constituents such as solvent-extractable soil organic matter). In FHoil layers, up to 39% (mean of 15%) of PHCs extracted from PHC-treated soils represented residual PHCs from the *in situ* soil contamination event 8 months earlier. No

significant changes in PHC concentration were apparent in mineral soil layers after 16 weeks, although levels in Ae layers tended to increase with time.

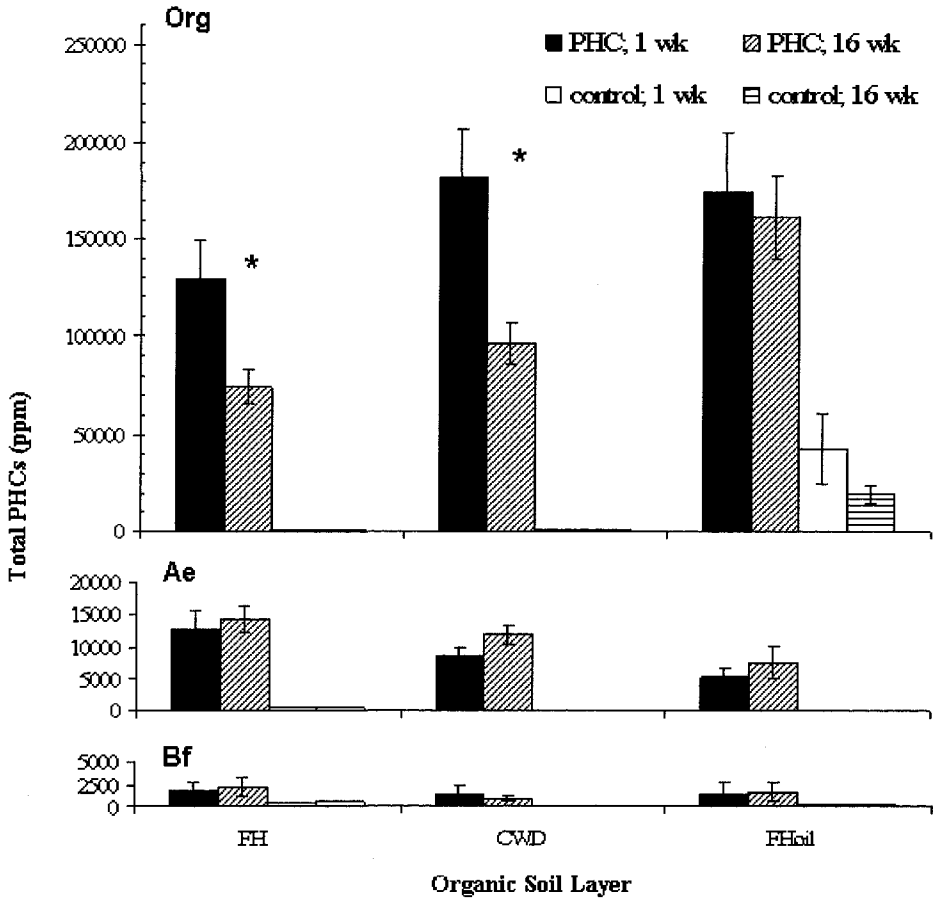

Figure 3.2: Concentration of total PHCs (ppm) in three organic soil layers (FH, CWD and FHoil) for PHC-treated and untreated controls at 1 and 16 weeks (data pooled for plant treatment). Bars represent standard errors of the means. Significant losses of PHCs within organic soil treatment groups are indicated by *.

Analysis of PHC fractions within plant – organic soil systems

Planted and unplanted systems were included in this analysis (i.e. P-FH, P-CWD, P-FHoil, B-FH, L-FH, L-CWD, and L-FHoil single-plant systems, PL-FH and PL-CWD double-plant systems, and N-FH, N-CWD, and N-FHoil no-plant systems). The F2 (nC10-nC16) fraction

PHCs from organic soil layers averaged 35,000 ppm after 1 week and 6,000 ppm after 16 weeks. Significant decreases in F2 PHCs were found in most planted and unplanted organic layers, including the P-CWD ($p=0.002$), B-FH ($p=0.02$), L-CWD ($p=0.03$), L-FHoil ($p=0.001$), N-FH ($p=0.02$), and N-CWD ($p=0.02$) treatments. The F3a (nC16-nC23) and F3b (nC23-nC34) fraction PHCs (containing straight-chain, branched and cyclic alkanes as well as small PAHs) were generally present between 40,000 and 80,000 ppm, but values varied greatly both within and between treatment groups (Figure 3.3). The F4 (nC34-nC50) fraction PHCs (containing some larger PAHs) were usually present in organic layers at <20,000 ppm. Although levels of larger PHC (>C16) chemicals also tended to decrease with time in organic soil layers, only P-CWD and B-FH systems showed consistently significant decreases in F3a ($p=0.001$ for both treatments), F3b ($p=0.004$ and 0.001, respectively) and F4 ($p=0.002$ and 0.009, respectively) fraction PHC concentrations over the study period.

In contrast to the organic layers, PHC concentrations tended to increase with time in Ae layers (perhaps due to leaching) (Figure 3.4). PHC levels in Ae layers under the CWD layer were extremely variable compared to levels in FH systems. F2, F3a, F3b and F4 fraction PHC levels were usually between 1,000 and 8,000 ppm; not high enough to account for all PHC loss from the organic layers above. Again, F4-fraction PHCs were the lowest (~1,000 ppm). For the Bf layers, PHC levels averaged about 1,000 ppm (Figure 3.5). PHC levels tended to increase most in the lingonberry compared to other plant systems.

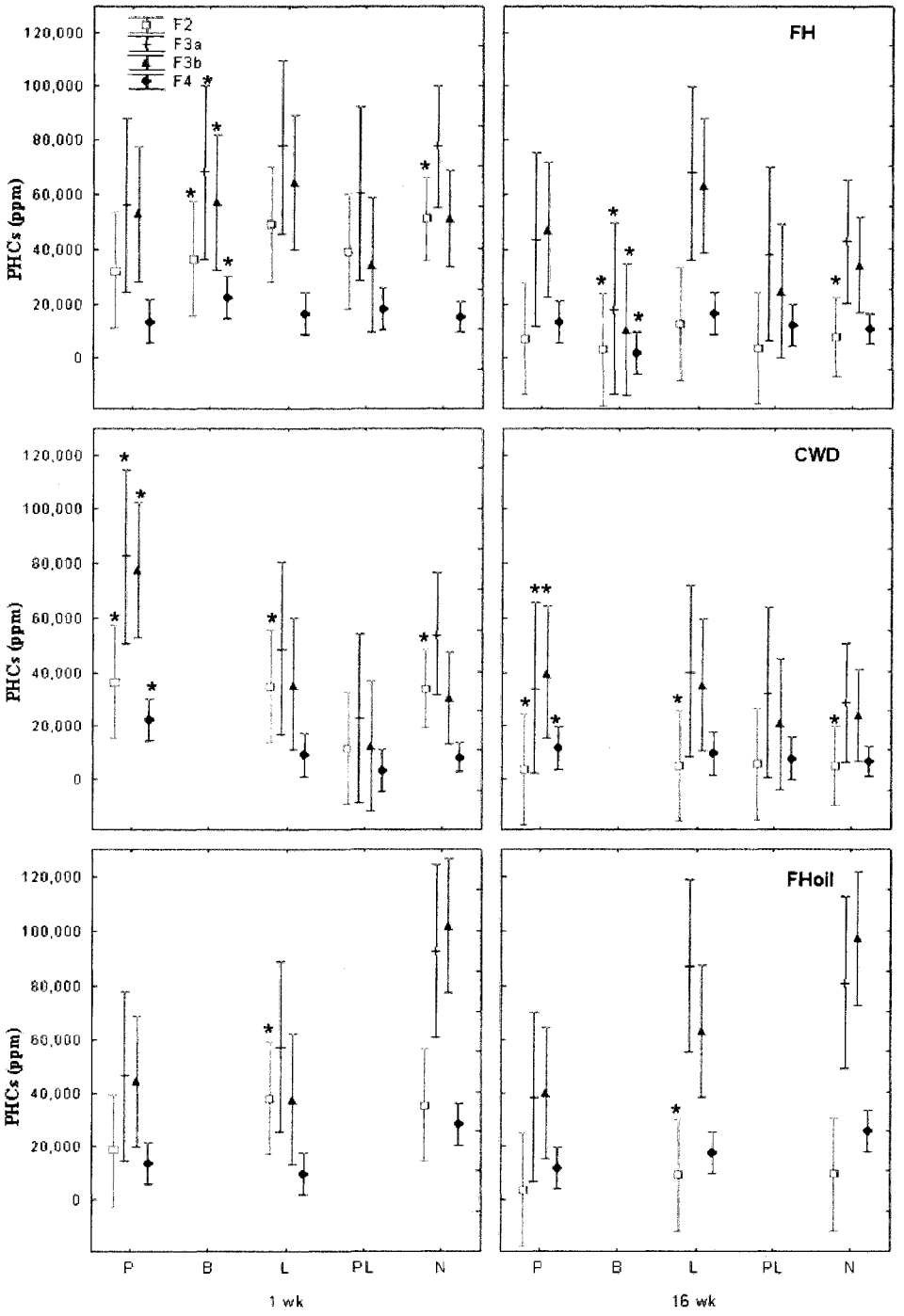

Figure 3.3: Concentrations of F2 (nC10-nC16), F3a (nC16-nC23), F3b (nC23-nC34), and F4 (nC34-nC50) PHCs (ppm) in organic layers of plant – soil systems at 1 and 16 weeks after PHC treatment. Plant treatments include: pine (P), birch (B), lingonberry (L), pine and lingonberry (P+L), and no plant (N). Bars represent standard errors of the means (n=3); significant differences (1-way ANOVA) within treatment groups are indicated by *.

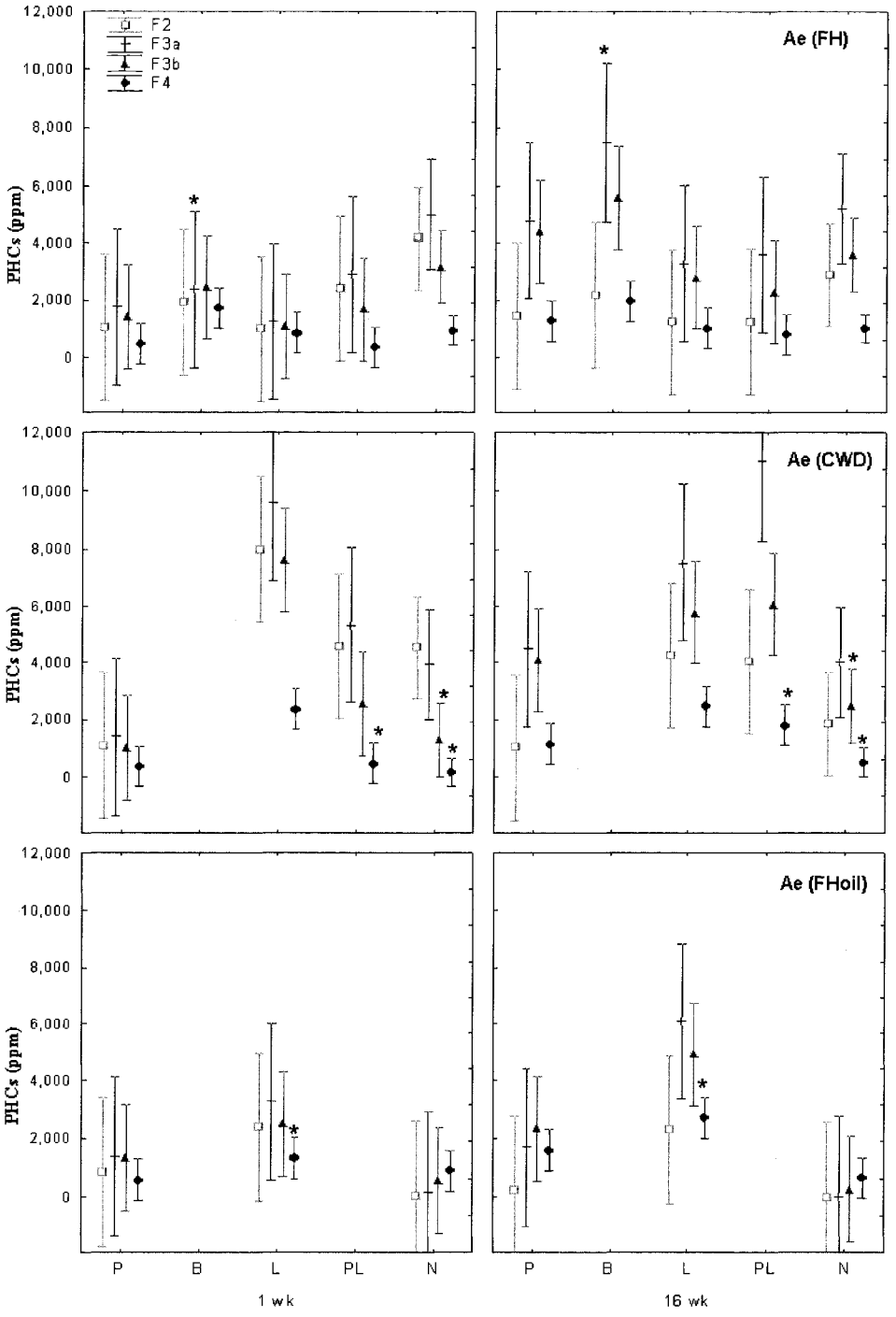

Figure 3.4: Concentrations of F2 (nC10-nC16), F3a (nC16-nC23), F3b (nC23-nC34), and F4 (nC34-nC50) PHCs (ppm) in Ae layers of plant – soil systems at 1 and 16 weeks after PHC treatment. Plant treatments include: pine (P), birch (B), lingonberry (L), pine and lingonberry (P+L), and no plant (N). Bars represent standard errors of the means (n=3); significant differences (1-way ANOVA) within treatment groups are indicated by *.

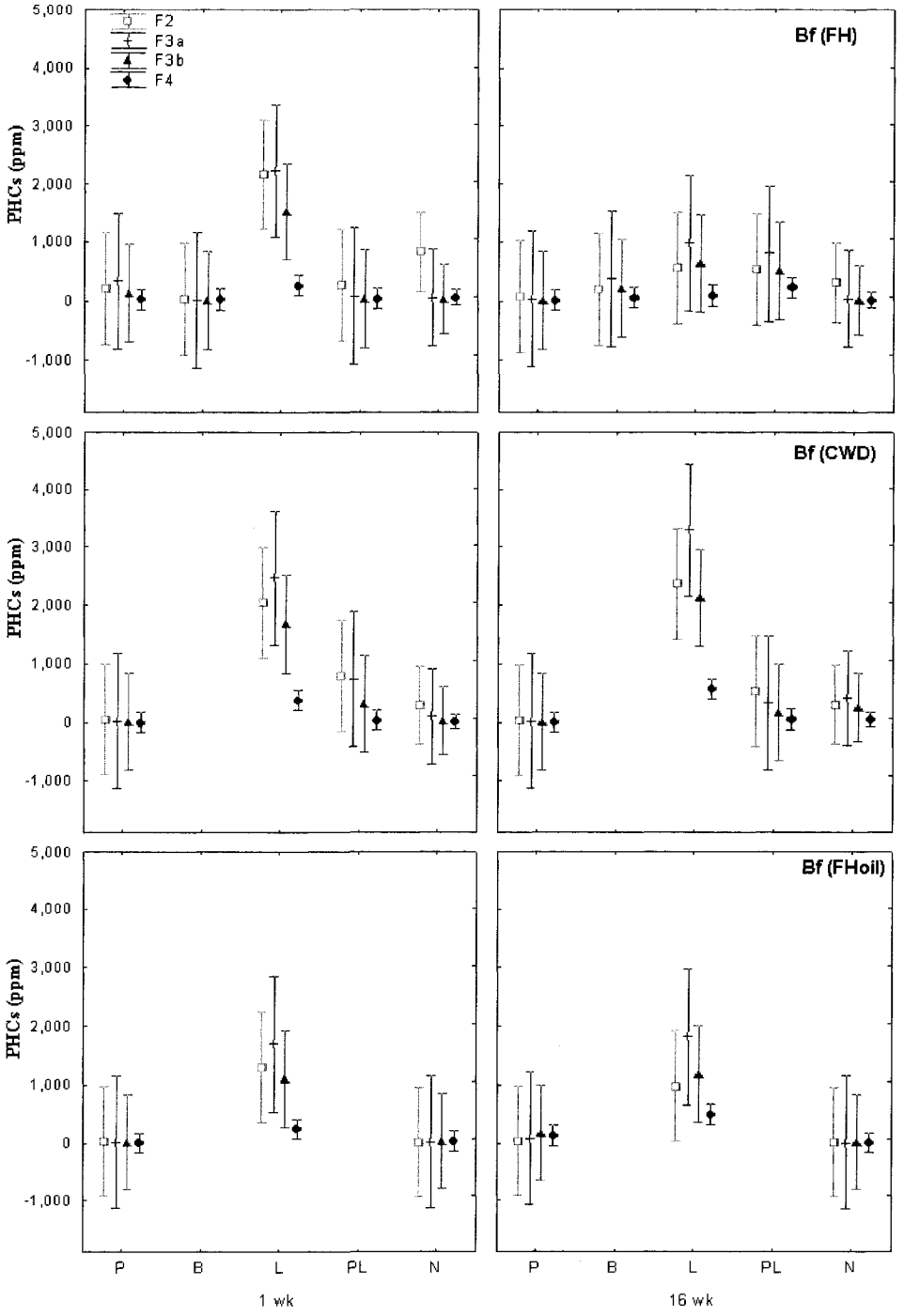

Figure 3.5: Concentrations of F2 (nC10-nC16), F3a (nC16-nC23), F3b (nC23-nC34), and F4 (nC34-nC50) PHCs (ppm) in Bf layers of plant – soil systems at 1 and 16 weeks after PHC treatment. Plant treatments include: pine (P), birch (B), lingonberry (L), pine and lingonberry (P+L), and no plant (N). Bars represent standard errors of the means (n=3); significant differences (1-way ANOVA) within treatment groups are indicated by *.

Soil properties

The C and N contents were much greater in organic (FH, CWD, or FHoil) compared to mineral (Ae or Bf) soil layers (Table 3.2). In organic layers, C content ranged from approximately 19-48% (mean C content of mineral soils was <2%) and was significantly greater ($p<0.001$) in PHC-treated soils compared to controls at both 1 and 16 weeks. The CWD layers contained significantly more C ($p<0.001$) than either of the FH or FHoil layers. The C content tended to be greater in FHoil compared to FH layers, presumably due to C inputs from the earlier PHC contamination event. Similar C levels were observed for all mineral soils at 1 week, but after 16 weeks, C levels tended to be greater in mineral soils below the PHC-treated organic layers, including the FHoil controls.

Total N ranged from approximately 0.28-0.64% in organic layers and generally did not vary with PHC treatment or over time. In contrast, mean N content of mineral soils was usually <0.1%. For both PHC-treated and control systems, N was significantly lower ($p<0.001$) in CWD soils compared to FH and FHoil soils at 1 and 16 weeks; mean N content of CWD was approximately half that of FH and FHoil soils. The combination of greater C and lower N content in CWD resulted in high C:N ratios that ranged from approximately 135 to183 and were significantly greater ($p<0.001$) than the C:N ratios in FH and FHoil soils, which were similar (i.e. ranging from approximately 30 to 65). Overall, C:N ratios did not change much between 1 and 16 weeks.

Comparison of planted and unplanted groups (data not shown) generally showed no differences in N and C content in organic or mineral soil layers, although, in Ae soils, C

tended to increase from 1 to 16 weeks in planted compared to unplanted systems (as well as in PHC-treated compared to untreated systems). There were no C differences in the Bf soils for the different plant treatments.

Soil pH (water) was generally lower in the organic and Ae soils (means of 5.3 and 5.4, respectively) than the Bf soils (mean of 5.7) at 1 week, but tended to increase after 16 weeks; this trend was greater in the untreated controls compared to the PHC-treated soils. In FH and CWD controls, pH significantly increased in all three soil layers (organic, Ae, and Bf), but no significant changes occurred in the FHoil controls. In the FHoil soil systems, pH was generally lower than in the FH and CWD systems, particularly in the Bf layer. Comparisons of plant treatments revealed no significant differences.

Table 3.2: Means (± standard errors) for soil properties (C, N, pH) in control and PHC-treated plant – organic soil systems at 1 and 16 weeks. Percent C and N are reported on an air-dry basis.

Week 1	FH		CWD		FHoil	
	no PHC	**PHC-treated**	**no PHC**	**PHC-treated**	**no PHC**	**PHC-treated**
Total C %						
Org	19.07 ± 2.040	33.80 ± 3.751	40.9 ± 1.761	48.4 ± 2.544	23.1 ± 3.397	36.92 ± 1.911
Ae	1.04 ± 0.235	1.68 ± 0.235	1.46 ± 0.210	1.90 ± 0.210	1.07 ± 0.210	1.68 ± 0.210
Bf	1.09 ± 0.189	1.75 ± 0.189	1.37 ± 0.169	1.34 ± 0.169	2.27 ± 0.169	2.18 ± 0.169
Total N %						
Org	0.60 ± 0.062	0.59 ± 0.034	0.31 ± 0.024	0.28 ± 0.029	0.60 ± 0.094	0.57 ± 0.031
Ae	0.055 ± 0.003	0.058 ± 0.003	0.045 ± 0.003	0.040 ± 0.003	0.054 ± 0.003	0.061 ± 0.003
Bf	0.062 ± 0.004	0.071 ± 0.004	0.067 ± 0.004	0.060 ± 0.004	0.11 ± 0.004	0.10 ± 0.004
C:N Ratio						
Org	31.9 ± 1.142	56.9 ± 5.291	134.9 ± 11.44	182.0 ± 26.46	40.3 ± 1.768	64.4 ± 0.840
Ae	18.7 ± 3.873	28.7 ± 3.873	31.7 ± 3.464	46.7 ± 3.464	19.6 ± 3.464	27.6 ± 3.464
Bf	17.4 ± 1.755	24.6 ± 1.755	20.4 ± 1.569	22.8 ± 1.569	20.8 ± 1.569	21.5 ± 1.569
pH						
Org	5.5 ± 0.1	5.4 ± 0.1	4.9 ± 0.1	5.0 ± 0.1	5.5 ± 0.1	5.4 ± 0.1
Ae	5.2 ± 0.1	5.0 ± 0.1	5.3 ± 0.1	5.2 ± 0.1	5.3 ± 0.1	5.3 ± 0.1
Bf	5.5 ± 0.1	5.9 ± 0.1	5.8 ± 0.1	5.5 ± 0.1	5.4 ± 0.1	5.4 ± 0.1

Week 16	FH		CWD		FHoil	
	no PHC	**PHC-treated**	**no PHC**	**PHC-treated**	**no PHC**	**PHC-treated**
Total C %						
Org	22.4 ± 1.866	29.3 ± 5.228	47.8 ± 2.275	53.8 ± 1.750	21.7 ± 2.354	37.2 ± 3.596
Ae	0.89 ± 0.235	2.32 ± 0.235	1.09 ± 0.235	2.83 ± 0.235	1.41 ± 0.235	2.080 ± 0.210
Bf	1.42 ± 0.189	1.92 ± 0.189	1.53 ± 0.189	2.05 ± 0.219	2.11 ± 0.189	2.18 ± 0.169
Total N %						
Org	0.644 ± 0.073	0.563 ± 0.063	0.29 ± 0.047	0.30 ± 0.021	0.558 ± 0.056	0.574 ± 0.041
Ae	0.049 ± 0.003	0.051 ± 0.003	0.045 ± 0.003	0.050 ± 0.003	0.064 ± 0.003	0.062 ± 0.003
Bf	0.073 ± 0.004	0.081 ± 0.004	0.080 ± 0.004	0.079 ± 0.004	0.104 ± 0.005	0.101 ± 0.004
C:N Ratio						
Org	35.3 ± 1.807	51.7 ± 6.547	174.1 ± 28.83	183.3 ± 20.33	38.9 ± 1.358	64.4 ± 3.320
Ae	18.0 ± 3.873	45.7 ± 3.873	24.4 ± 3.873	56.4 ± 3.87	22.2 ± 3.873	33.1 ± 3.464
Bf	19.3 ± 1.755	23.7 ± 1.755	19.2 ± 1.755	25.7 ± 2.03	20.3 ± 1.755	21.4 ± 1.569
pH						
Org	5.6 ± 0.2	5.4 ± 0.1	5.4 ± 0.3	5.1 ± 0.2	5.3 ± 0.1	5.0 ± 0.1
Ae	5.8 ± 0.1	5.3 ± 0.1	5.7 ± 0.1	5.2 ± 0.1	5.6 ± 0.1	5.6 ± 0.1
Bf	5.9 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	6.0 ± 0.1	5.5 ± 0.1	5.6 ± 0.1

Bacterial community structure

Overall, PHC treatment of the plant – soil systems had no effect on genotypic richness or structure (composition) of bacterial communities. Pairwise comparisons (MRPP) of PHC treated systems at 1 and 16 weeks revealed that genotype richness increased significantly ($p=0.007$) over time. Community structure varied significantly ($p=0.036$) between PHC-treated and control groups after 16 weeks.

Analysis (NPMANOVA and MRPP) of the single-plant (i.e. pine, birch and lingonberry) systems showed significant differences ($p=0.002$) in bacterial community structure in the different organic soil layer treatments. Pairwise comparisons indicated that the differences were between FH and FHoil groups ($p<0.001$), and CWD and FHoil groups ($p=0.009$), but not between FH and CWD groups. There were no interaction effects between organic soil layer and PHC groups.

The type of plant (i.e. pine, birch or lingonberry) appeared to have less of an effect on genotype richness and community structure than the complexity (i.e. single- or double-plant) of the systems (Figure 3.6). The single-plant systems did not significantly differ with respect to either genotype richness or community structure. However, community structure varied significantly ($p<0.001$) for both pine and lingonberry in double- compared to single-plant systems; community structure also varied between pine and lingonberry within the double-plant systems. These patterns were consistent for the richness data, with an additional significant difference ($p<0.001$) between pine and lingonberry in the single-plant systems. There were no interaction effects between plant and either PHC, harvest, or organic soil layer

groups. The overall effect of plant on bacterial community structure is shown in the NMS ordination (Figure 3.7). The figure shows tight clustering (in the lower left quadrant) of communities from the double-plant systems within the more dispersed distribution of single-plant system communities.

Seven DNA fragments corresponded to 16S fragment lengths for *Pseudomonas* (343, 344 bp) and *Sphingomonas* (317, 318, 319, 320, and 335 bp) species (Mills *et al.*, 2003). The frequency of these fragments (nested one-way ANOVA) varied with plant, but did not vary over time or with organic soil layer or PHC treatment, although some genotypes increased or decreased in frequency with different plants.

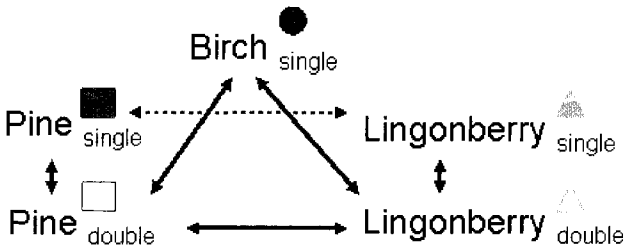

Figure 3.6: Schematic diagram showing plant effects on bacterial genotype richness and community structure. Arrows (solid, genotype richness and community structure; dashed, richness only) between plant treatments indicate significant differences. Treatments not connected by arrows are not significantly different.

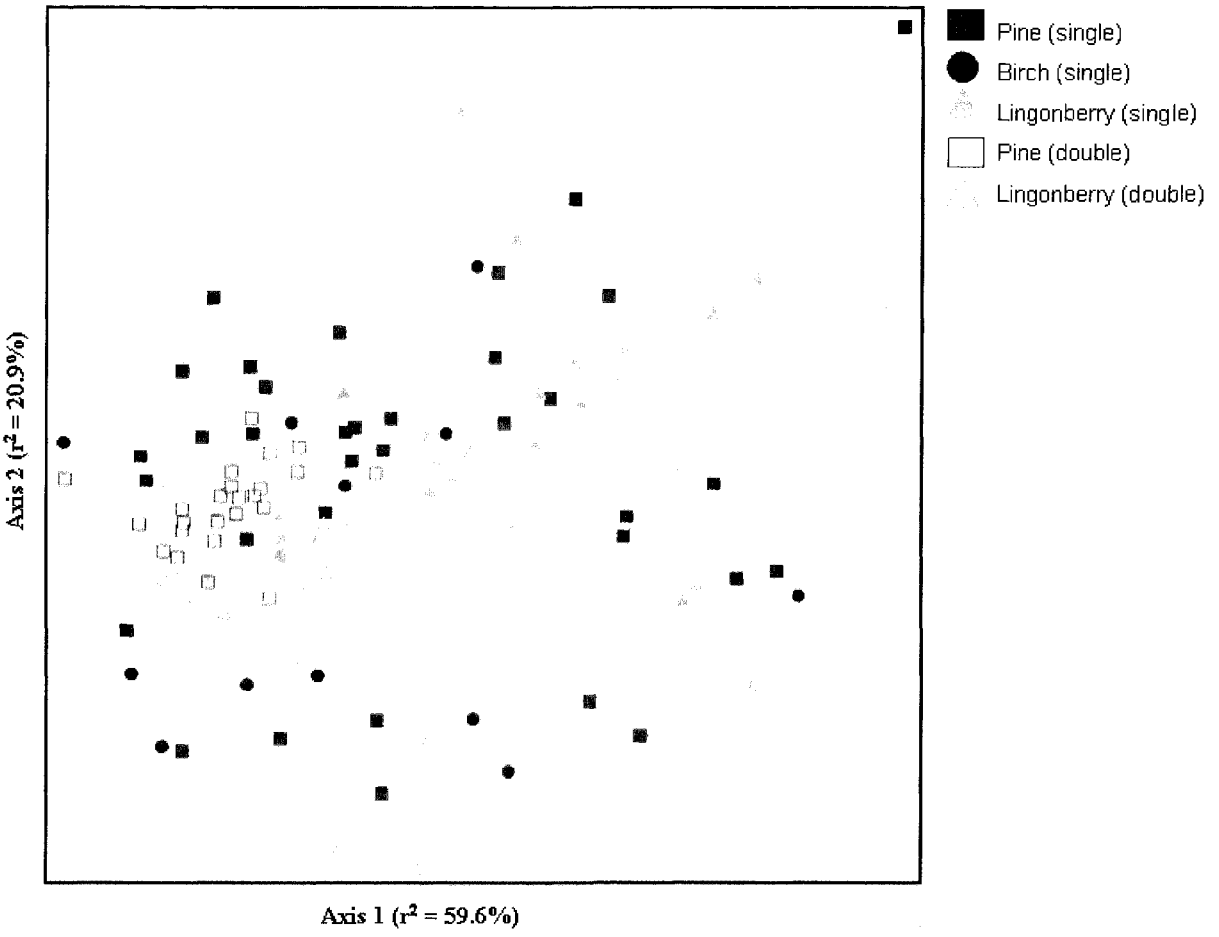

Figure 3.7: NMS of bacterial community structure associated with single- (pine, birch or lingonberry) and double- (pine + lingonberry) plant systems (stress = 16.42; instability = 0.06).

Bacterial community level physiological profiles

Areas under C substrate oxidation curves for bacterial communities growing on EcoPlates™ were consistently greater for amino acids, carbohydrates and carboxylic acids in all treatments; lower values were observed for amides/ amines, polymers and the miscellaneous substrates. No differences in substrate use were found between PHC-treated and untreated communities or within the different organic soil groups. In the unplanted FH and CWD systems, areas under the substrate use curves were lower in the PHC-treated compared to untreated systems (which were similar to the planted system profiles); in the unplanted FHoil systems, areas under the curves were virtually identical in the PHC-treated and control soils and both treatments produced profiles similar to the planted system profiles.

Principal components analysis (PCA) of the areas under substrate use curves (24-120 h) for the various plant – organic layer – PHC treatments generated the ordination shown in Figure 3.8; the two principal components axes explained 85.02% of the variation between samples. The N-containing substrates (amines/ amides and amino acids) showed the greatest relative effect on the plot, followed by carbohydrates and carboxylic acids. Of the treatment variables, plant exhibited a greater effect than either organic soil or PHC. There was a tendency for unplanted systems to group away from the planted systems (i.e. towards the right side of the plot); the lingonberry treatments also grouped slightly away from pine, birch and alder (i.e. towards the lower quadrants of the plot).

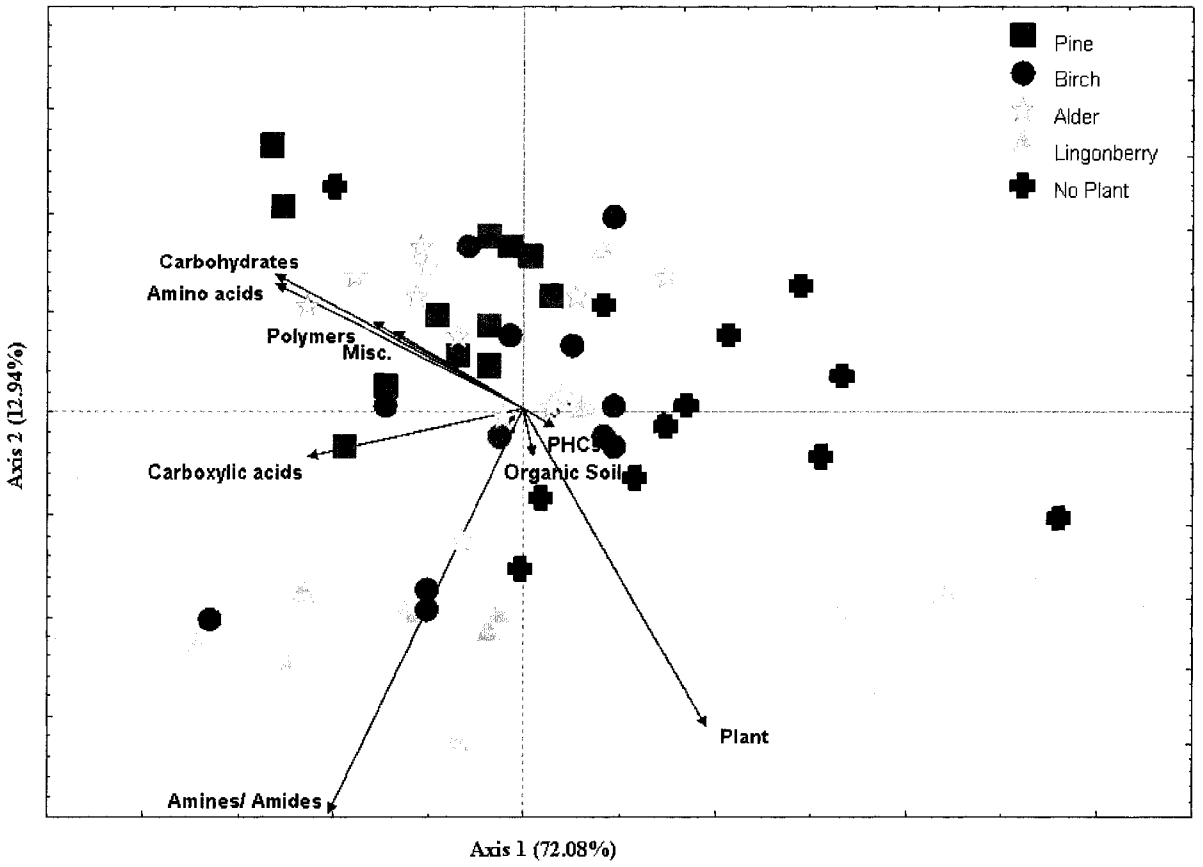

Figure 3.8: PCA ordination of areas under C substrate curves showing the relative influence of PHC, organic soil and plant treatment variables. Plant effects are indicated by different symbols: pine (squares), birch (circles), alder (stars), lingonberry (triangles), and no plant (crosses).

Discussion

PHC Biodegradation

The process of PHC biodegradation in reconstructed sub-boreal forest soil systems was more closely related to qualities of the organic soil layer than to genotypic or broad metabolic structure of the bacterial communities in the mycorrhizosphere. We found significant reduction of total PHC levels following (1-16 weeks) addition of crude oil to pristine forest floor (FH) and coarse woody debris (CWD) soil systems. These lignin-rich and metabolically active soil layers form substantial surface components of northern forest landscapes (Lundström *et al.,* 2000; Prescott *et al.,* 2000). Their tendency to retain PHCs in the soil organic matter was evident in our study, as PHC concentrations were far greater than those extracted from the underlying mineral soils in each system. Soil analyses showed a significantly lower pH and higher C:N ratio in CWD compared to FH layers at both 1 and 16 weeks; however, these differences did not appear to reduce biodegradation capacity of these soil communities. The intrinsic ability for PHC biodegradation existed within soil microbial communities of the pristine organic layers.

Prior *in situ* treatment of the forest floor with PHCs did not appear to have enhanced microbial biodegradation capacity for subsequent PHC treatment in these soils, as was expected (Miller and Herman 1997; Braddock *et al.,* 2003; Seghers *et al.,* 2003; Díaz 2004). The lack of significant PHC reduction over the study period found in the previously contaminated (FHoil) soil layer may be partly explained by the presence of residual PHCs. Residual PHCs likely consisted of a greater proportion of more recalcitrant compounds that contributed to a more chemically complex and heterogeneous environment. Aging of

contaminants in soil generally reduces bioavailability to decomposer organisms (Alexander, 2000). This may have increased variation in biodegradation patterns within treatment groups and masked any significant PHC loss over the 16-week duration of the experiment. The C:N ratio of FHoil soils was not significantly greater than FH soils; neither differed substantially from the C:N ratios reported for the forest floor measured *in situ* (about 50) (Arocena and Sanborn, 1999) or from oil-contaminated soils in Alaska (52.7) (Braddock *et al.,* 2003). The pH of FHoil was similar to CWD; both were lower than FH, particularly after 16 weeks. These physico-chemical soil properties were therefore not related to the differences in biodegradation observed among the different organic soil layers. Although FH and CWD exhibited significantly different bacterial community structure patterns compared to FHoil layers, no differences in C use profiles were found.

Processes that reduce PHC concentrations in oiled soils over time (i.e. biodegradation, evaporation, water washing, or photooxidation) lead to distinct chemical profiles in soil; a decrease in n-alkane (<nC17) concentration relative to larger compounds and PAHs is typical of biodegradation profiles (Braddock *et al.,* 2003). In general, we found a trend of reduced F2 (nC10-nC16) PHCs for all treatment groups by 16 weeks. Several plant - soil systems exhibited significant decreases in F2 concentrations, including both planted (birch -FH, pine - CWD, lingonberry -CWD and -FHoil) and unplanted (-FH and -CWD) systems. This finding was not surprising as many genera of soil bacteria are known to completely mineralize aliphatic compounds (e.g. alkanes <nC16) through central metabolic pathways such as β-oxidation and Kreb's cycle (McGill *et al.,* 1981; Miller and Herman 1997). Our results suggest that biodegradation capacity for F2 PHCs exists within all the soil systems tested.

Also, unplanted soils, which originated as sub-boreal forest rhizosphere, continued to support biodegradative microbial communities for at least eight months after soil collection.

In contrast, significant decreases of F3a (nC16-nC23), F3b (nC23-nC34) and F4 (nC34-nC50) PHCs occurred only in pine -CWD and birch -FH systems. Enhanced PAH degradation in rhizosphere compared to non-rhizosphere soils was also reported by Joner *et al.* (2006). Microbial utilization of PHCs often creates demand for other nutrients such as N and P (Miller and Herman, 1997). Thus, it is likely that the energy (C) and nutrient (N and P) resources necessary to support biodegradation of these larger and more chemically complex PHCs were limited in the unplanted soil systems that lacked inputs of C-rich substrates through exudation from mycorrhizal roots. Strong sorption of these larger PHC compounds to soil components may have also reduced their bioavailability to degrading microorganisms. Alternatively, the lack of significant change in levels of larger PHCs may have been due to slow decomposition of these compounds (i.e. perhaps 16 weeks was not long enough to detect changes in PHC concentrations).

Mycorrhizosphere effect

The mycorrhizosphere generally supports greater microbial biomass and activity (i.e. mycorrhizosphere effect) than non-rhizosphere soils, which increases the biodegradative potential of planted over unplanted systems (Linderman, 1988; Ingham and Molina, 1991; Rygiewicz and Anderson, 1994). In PHC-contaminated soils, plant-fungal exudates are also expected to support proliferation of functional groups of organisms with enhanced capacity for cometabolic biodegradation of PHCs. With respect to pyrene biodegradation, Mueller

and Shann (2007) showed that the quality of C in root exudates was more important than C quantity, suggesting that specific mycorrhizosphere communities may influence biodegradation. Several studies have reported elevated densities of PHC-degrading bacteria in contaminated soils (Lindstrom *et al.,* 1999; Delille *et al.,* 2004). Braddock *et al.* (2003) found that culturable populations of total heterotrophic and crude oil emulsifying bacteria were elevated in soils 25 years after an Alaskan oil spill compared to soils from an adjacent reference plot. Our comparisons of bacterial community C use profiles distinguished unplanted from planted soils on the PCA ordination. Palmroth *et al.* (2005) also showed C utilization differences between vegetated (pine and poplar) and unvegetated soil communities using similar methods. In our study, nested analyses within organic soil and PHC treatment groups showed lower metabolic activity in only the untreated FH and CWD systems; preliminary plate counts (data not shown) also indicated lower culturable bacterial density in untreated (i.e. no PHC added) FH and CWD (10^4-10^5 CFUs mL^{-1} soil solution) compared to either the untreated FHoil or the PHC-treated FH, CWD and FHoil (10^7-10^8 CFUs mL^{-1} soil solution) systems. These findings suggest that C inputs by both plants and PHCs support greater bacterial density; however, a plant appears to enhance biodegradation of >nC16 PHCs.

The finding that statistically significant reductions of the larger PHC (nC16+) fractions occurred in only planted (pine and birch) systems provides indirect evidence for a stimulatory role of ECMs in the biodegradation process. ECMs may simply provide habitat and C substrates that enhance bacterial cometabolism, as described earlier (Sarand *et al.,* 1998; 2000; Heinonsalo *et al.,* 2004). In addition, ECMs may secrete oxidative enzymes that

open aromatic ring structures, thereby overcoming the thermodynamically limiting step in PAH metabolism (Burke and Cairney, 2002; Sen, 2003). Several recent studies have reported that mycorrhizas inhibited biodegradation of naphthalene, fluorene (Genney *et al.,* 2004), anthracene, anthraquinone, chrysene, dibenz[a,h]anthracene (Joner *et al.,* 2006), and pyrene (Koivula *et al.,* 2004) when individually spiked into microcosms. Genney *et al.* (2004) suggested that ECMs inhibit PHC biodegradation in situations of C limitation. Thus, in these studies, PAH treatment in the absence of cometabolic substrates may have led to increased competition between fungi and bacteria for the same energy resources, resulting in decreased PAH mineralization and accumulation of dead-end metabolites in soil (Gadgil and Gadgil, 1971).

Differences in experimental design may explain our findings in context with these studies. In our study the establishment of diverse ECM communities on the fine roots of pine and birch was observed to be virtually ubiquitous, which likely reduced free-living soil fungi in the rhizosphere and thus limited this group of competitors (Lindahl *et al.*, 2007). Incorporation of soil depth (i.e. increased three-dimensional space in miniature soil systems that contained horizons) in our mycorrhizal systems was also expected to reduce competition between ECM fungi with saprotrophic activities and the associated bacterial community (Cairney, 2005). Also, addition of reduced C substrates in PHC mixtures probably fueled the large heterotrophic biomass associated with the pine and birch mycorrhizospheres (Nannipieri *et al.,* 2003; Morgan *et al.,* 2005), resulting in a generally stimulatory effect on biodegradation. This explanation is supported by Koivula *et al.* (2004), who found that inhibition of PHC biodegradation (due to C limitation) was eased in oiled soils where alternate C substrates

were available. However, an overall inhibitory effect on biodegradation could explain why most mycorrhizal systems (including ERM systems) did not exhibit significant losses of larger PHCs in the current study.

Although ERMs have been reported to exhibit oxidative enzyme activity (Burke and Cairney, 2002), the lack of significant loss of larger PHCs indicates that they did not contribute to biodegradation in our lingonberry systems. One explanation may be that the fine root systems were shallow and lacked extensive ERM mycelia that did not sufficiently fill the larger pots into which the single lingonberry seedlings were planted; secreted enzymes and exudates may have been diluted with distance from the ERM roots. Joner and Leyval (2003) reported that the (mycor)rhizosphere effect on PAH degradation sometimes does not extend more than 1 mm from roots colonized by arbuscular mycorrhizas. Corgié *et al.* (2003) described bacterial gradients with increased densities of heterotrophs and PAH degraders closest to the roots (0-3 mm) that corresponded to a gradient of phenanthrene biodegradation. In a molecular study, Corgié *et al.* (2006) showed different bacterial communities were selected by rhizosphere depending on distance from the roots; bacteria exhibited different activity profiles and values for biodegradation. Alternatively, it is possible that lingonberry roots were not mycorrhizal (i.e. cell colonization, which was confirmed by microscopy, may not have included ERM fungi), although we assumed that ERM fungi accounted for at least some of the endophytic community. Very little is known regarding biodegradation capacity in ERM systems, and further investigation is warranted.

In the single-plant systems examined here and by Palmroth *et al.* (2005), C use profiles showed little differentiation between bacterial communities associated with the mycorrhizosphere of specific plants, although lingonberry appeared to cluster slightly away from pine, birch and alder. Bacterial genotype richness was significantly greater in lingonberry compared to pine and birch systems. It is unknown whether this greater richness is somehow related to the significant biodegradation for the F2 PHC fraction observed in lingonberry -CWD and -FHoil systems. Bacterial community structure varied significantly for both pine and lingonberry in the double-plant systems compared to any of the single-plant systems. Furthermore, bacterial community structure varied significantly between lingonberry and pine when the two plants shared the same pot. The NMS analysis graphically reaffirms that growth conditions between double- (tight clustering) and single- (more dispersed distribution) plant systems are different enough to influence bacteria community structure. The soil conditions being the same, we presume that a double-plant system augments the level of competitive interactions (perhaps by providing more specialized niches owing to different root systems) compared to a single-plant system, and that is reflected in the bacterial diversity. Although significant PHC biodegradation was not observed in either of the double-plant (pine and lingonberry -FH or -CWD) systems tested, this may represent another example of increased environmental complexity where within-group variation was too great to detect PHC reduction over 16 weeks.

It was unfortunate that difficulties with seed germination resulted in incomplete datasets for the alder treatment. Associations with N-fixing *Frankia* were expected to enhance PHC biodegradation via a gradual and continuous delivery of N and C to the rhizosphere

environment (Selmants *et al.,* 2005; Roy *et al.,* 2007). A hint of the effect of greater N availability on mycorrhizosphere communities (i.e. altered amino acid requirements) may have slightly influenced the C use distribution of alder compared to the other ECM systems. Interactions between this additional functional group and the rest of the mycorrhizosphere community should be investigated in future PHC biodegradation studies.

Overall, surface application of crude oil appeared to have minimal impact on the diversity (genotype richness and relative abundance) or metabolic capacity (community physiological profiles) of bacterial communities in the mycorrhizosphere. Huertas *et al.* (2000) suggested that heterogeneity in the soil matrix may provide a protective effect against the solvent shock associated with initial PHC contamination. Other studies have also reported the inability to distinguish communities from PHC-contaminated and reference soils on the basis of broad physiologies such as C mineralization; bacterial communities surviving in oiled soils have been described as metabolic generalists (Lindstrom *et al.,* 1999; Palmroth *et al.,* 2005). However, the physiological capacity for biodegradation of various organic substrates, which is controlled by the functional redundancy of the community originally present (Setälä *et al.,* 2000; Delille *et al.,* 2003), seems to remain intact in PHC-contaminated soils for some time in the absence of further disturbance.

The methods used in this study did not distinguish the PHC-degrading component from the overall composition of bacterial communities. However, common hydrocarbon degraders such as *Pseudomonas* sp. grow well on Biolog EcoPlates^{TM} and thus were expected to be represented as part of the C-utilizing community assessed. The molecular dataset was also

expected to include known PHC-degraders (e.g. pseudomonads and sphingomonads) whose corresponding fragment lengths were identified (i.e. 342-344 bp and 317-320, 334-335 bp, respectively) by Mills *et al.* (2003) using the same rDNA primer pair. These fragments appeared in both PHC-treated and control mycorrhizospheres of pine, birch and lingonberry systems, with no differences in frequency observed from 1-16 weeks.

Conclusions

We found that mycorrhizosphere communities enhanced PHC biodegradation in reconstructed sub-boreal forest soils, particularly in ECM systems. The ECM and ERM fungal communities associated with these systems and their roles in the biodegradation process were investigated in Chapter 2. The level of PHC contamination used in this study appeared to have minimal impact on soil bacterial community structure or broad metabolic functions associated with PHC biodegradation.

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Chapter 4: Root-associated microbial communities differ with *Pinus contorta* var. *latifolia* and *Vaccinium vitis-idaea* co-inhabiting sub-boreal forest soils

Abstract

Rhizosphere communities, including mycorrhizas and closely associated heterotrophic microorganisms, represent critical functional groups for decomposition and carbon/ nutrient cycling processes in northern forest ecosystems. The spatial heterogeneity of the soil environment contributes to the great biodiversity of soil microorganisms. However, the complexity of the system and the multifunctional nature of many microorganisms have made it difficult to comprehend linkages between communities and ecosystem processes and also to predict how ecosystems may respond to environmental disturbances such as soil contamination. In this study, we used a bioassay approach to assess the relative contributions of plant and soil properties to spatial distribution patterns of ecto- (ECM) and ericoid (ERM) mycorrhizal fungi as well as root-associated bacterial communities inhabiting the shared rhizosphere of pine (ECM host) and lingonberry (ERM host). Soil systems were either untreated or treated with petroleum hydrocarbons (PHCs), simulating contamination events. Surface-sterilized pine (*Pinus contorta* var. *latifolia*) seeds and lingonberry (*Vaccinium vitis-idaea*) seedlings were planted into Conetainer^{TM} pots containing an organic layer (mor humus [FH] or coarse woody debris [CWD]) overlying sandy mineral horizons (Ae and Bf) of field-collected forest soils obtained from central BC, Canada. After 4 months, BC light crude oil (219 mg cm^{-2}) was applied to the soil surface around the seedling stem; systems were destructively sampled at 1 and 16 weeks following treatment. Soils from each layer were analyzed for PHC concentration (not presented here), pH and total C and N content. The composition, relative abundance and vertical distribution (i.e. variation with soil layer)

of eight frequently occurring ECMs on pine roots were assessed using light microscopy. Community profiles (i.e. based on the relative abundance of all genotypes) were generated for all root systems using length heterogeneity PCR and primers targeting the ITS (fungi) and 16S (bacteria) regions of ribosomal DNA. We found that the main components of ECM communities were consistent with those described from field-based studies. Genotype analysis by non-metric multidimensional scaling (NMS) and multi-response permutation procedures (MRPP) revealed that both plant and soil properties influenced the structure of root-associated fungal and bacterial communities; however, patterns of community structure varied among the different functional groups. Fungal communities were distinctly different on pine (ECM) and lingonberry (ERM) roots; only ECM fungal communities were structured vertically in the three layers of soil, representing direct interactions between fungi and soil in the ectomycorrhizal association. In contrast, ERM communities appeared to vary more between soil systems (i.e. FH-Ae-Bf and CWD-Ae-Bf) than between soil layers. Bacterial community structure varied between mycorrhizal root systems and between soil layers, indicating that differences between the ECM and ERM root environment and soil properties are both important with respect to bacterial niche differentiation. PHC contamination appeared to have little effect on the composition of root-associated microbial communities.

Introduction

Rhizosphere communities, including mycorrhizas and closely associated heterotrophic microorganisms, represent critical functional groups for decomposition and carbon/ nutrient cycling processes in northern forest ecosystems. The spatial heterogeneity of the soil environment contributes to the great biodiversity of soil microorganisms and promotes species co-existence through greater resource partitioning (Ettema and Wardle, 2002; Ramette and Tiedje, 2007). However, the complexity of the system and the multifunctional nature of many microorganisms have made it difficult to comprehend linkages between communities and ecosystem processes and also to predict how ecosystems may respond to environmental disturbances such as soil contamination (Barrios, 2007). Disturbances may directly impact microbial functions, lead to loss of a functional group, change rates of ecosystem processes or alter resource availability (Balser *et al.*, 2006). The question arises as to how much detail is necessary to understand system behaviour and to mitigate for effects of environmental disturbance (Surridge, 2006). To answer this question may require that we step back from studying individual system components in favour of studying functional groups of soil biota that have tight linkages to functions that underpin soil-based ecosystem services (Balser *et al.*, 2006; Smithwick, 2006; Barrios, 2007).

Northern forests are dominated by trees in the families Pinaceae, Betulaceae, Fagaceae and Salicaceae that typically form ectomycorrhizal (ECM) symbioses with soil fungi that exhibit variable distribution patterns across forest landscapes according to host specificity (Molina *et al.*, 1992) and soil heterogeneity (Horton and Bruns, 2001). ECMs occupy the structural and functional interface between the plant and soil environment and regulate plant nutrient uptake

as well as carbon release to soil (Smith and Read, 1997). These mechanisms of carbon and nutrient exchange are understood to some extent (i.e. at genetic and biochemical levels) in ECM systems, but not in an ecological context, which involves interactions with other microbial communities sharing the rhizosphere. Although interactions with heterotrophic bacterial communities associated with the ECM mantle and extraradical mycelia are known to be important for mobilization, uptake and translocation of nutrients (Burke and Cairney, 1998), an ecological understanding is impeded by that lack of integration between ECM fungal and bacterial studies. Additionally, roots of understory vegetation such as Ericaceae (e.g. *Vaccinium*, *Rhododendron, Gaultheria*, species) commonly share ECM rhizosphere space (Read and Perez-Moreno, 2003). These plants usually form ericoid mycorrhizas (ERMs) with fungi that are often unidentified, but also appear to exhibit high diversity at small scales (Berch *et al.*, 2002; Perotto *et al.*, 2002) and are expected to contribute to nutrient cycling and decomposition processes in forests (Cairney, 2000; Read and Perez-Moreno, 2003). Interactions between different mycorrhizal communities, as well as potential sharing of fungal symbionts, may contribute to ecological processes in important ways that we do not yet understand.

Petroleum hydrocarbon (PHC) contamination is a type of environmental disturbance that can lead to considerable changes in physical and chemical soil properties on spatial scales ranging from square meters to hectares. Initially, spilled PHCs spread laterally within the organic layer of forest soils; lighter fractions eventually move down the soil profile, along the paths of roots and fissures. Fragmentary patterns of PHC constituents that persist in mobile form, fixed in soil pores and fissures, adsorbed on the surface of organic and mineral soil

constituents, or forming a free-phase continuous cover on the soil surface (Trofimov and Rozanova, 2003) increase soil heterogeneity and have unknown effects on soil microbial communities. The major impacts appear to be related to altered soil water, nutrient and oxygen regimes (Tarradellas and Bitton, 1997). At the same time, input of labile C substrates (and subsequent release of soil communities from C limitation) may promote metabolic activity on the part of all the microorganisms not directly inhibited by the PHCs and provide the potential for them to play a major role in decomposition and nutrient mobilization (Read and Perez-Moreno, 2003). The unevenness of PHC distribution in different soil layers may impact different microbial communities in contrasting ways that could reveal new features of functional diversity in forest soils.

In this study, we used a bioassay approach to assess the relative contributions of plant and soil properties to spatial distribution patterns of ecto- (ECM) and ericoid (ERM) mycorrhizal fungi as well as root-associated bacterial communities inhabiting the shared rhizosphere of pine (ECM host) and lingonberry (ERM host). Soil systems were either untreated or treated with PHCs to gain some understanding of how communities may change in response to PHC contamination events. Using a combination of morphological and molecular methods to describe patterns of community structure along with multivariate analysis techniques to correlate soil properties, we aimed to contribute to the understanding of ecophysiological functioning of this group of microorganisms that is ubiquitous on the northern forest landscape.

Materials and methods

Field site

In September of 2006, forest soils were collected from the Kenneth Creek field site, located about 100 km east of Prince George (53°34'N, 122°47'W) in the wet, cool subzone of the sub-boreal spruce (SBSwk1) biogeoclimatic zone of central British Columbia. In 1982, the forest was logged and burned, then subsequently planted with lodgepole pine (*Pinus contorta* Dougl. Ex Loud. var. *latifolia* Engelm.); currently, the site is a mature, even-aged pine stand with small hybrid white spruce (*Picea glauca* x *engelmannii* Parry *ex* Engelm.) and a few western hemlock (*Tsuga heterophylla* (Raf.) Sarg.). Young subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.) and sitka alder (*Alnus crispa* var. *sinuata* (Reg.) Rydb.) are present at the edge of the forest, along the access road; western redcedar (*Thuja plicata* Donn) and trembling aspen (*Populus tremuloides* Michx.) are also present in an unlogged stand across the main road. The site has a thick understory of oval-leaved blueberry (*Vaccinium ovalifolium* Sm.); mosses and lichens (e.g. *Peltigera*) cover the forest floor, with some *Lycopodium* species.

The soils on this site were described by Arocena and Sanborn (1999). Soils are classified as Eluviated Dystric Brunisols (Soil Classification Working Group, 1998), and consist of sandy parent material with low clay content and few coarse fragments. The forest floor is mor humus, from 2-5 cm thick with copious fungal mycelia present. The C:N ratio of the forest floor is approximately 50 and the pH (water) is approximately 4.2. Gray Ae horizons are generally 1-2 cm thick, with thicker pockets in some areas. Red Bf horizons extend to almost 30 cm, beneath which are Bm (27-60 cm), BC (60-100) and C (> 1 m) horizons. The C:N ratios of the Ae and Bf horizons are about 20 and 13, respectively. The pH (water) of the Ae

horizon is 4.2 while the pH of the Bf horizon is about 4.8, increasing with depth to about 6.0 at the transition to the C horizon. Fine roots are found at depths greater than 1 m. Large coarse woody debris (i.e. downed trees), the legacy of past forest management, is abundant all over the site.

Bioassay, PHC treatment and sampling

Forest soil layers were reconstructed (i.e. Ae [~1 cm] and Bf [~15 cm] mineral soil layers beneath organic [FH or CWD] soil layers [~2 cm]) in Cone-tainer^{TM} pots (3.8 x 21 cm, Stuewe and Sons, Corvalis, Oregon), as described previously. Lingonberry (*Vaccinium vitis-idaea* L.) seedlings were transplanted into these soils and surface-sterilized seeds of lodgepole pine (*Pinus contorta* var. *latifolia*), collected from the SBS and obtained from the Ministry Tree Seed Center, Surrey, BC (seed lot DWD20050009A (location 079-B-008)), were then planted into the organic layer of each pot. All pots were kept in the greenhouse (22°C day temperature, 15°C night temperature, and16 h photoperiod) and watered two or three times per week for the duration of the experiment. The plants were fertilized once a month (5 mL of NPK fertilizer; providing 100 ppm each of NPK) for the first four months during seedling and mycorrhizal (ECM or ERM) establishment. At four months, 3 mL crude oil (219 mg cm^{-2}) was pipetted onto the organic soil surface of each pot, around, but not touching, the seedling stem.

Plant – soil systems were destructively sampled at 1 and 16 weeks following PHC treatment. Individual pots were emptied into trays, and the two plants (pine and lingonberry) were gently removed from the soil with as little disturbance as possible to the three soil layers

(organic, Ae and Bf). Soil layers were collected from each pot, combined within treatment groups (Table 4.1), and then air-dried for soil nutrient analysis and pH. PHC concentrations in soil layers were also determined and are presented in Chapter 3. Root systems (with the pine or lingonberry seedling excised) were shaken free from the soil and washed in sterile dH_2O. All root systems were examined under a dissecting microscope to ensure that they were free of other roots or hyphae and soil particles; the distribution of ECM morphotypes was also described for pine root systems. Root systems were then divided into three samples (organic, Ae and Bf soil layers) and stored in 2 mL tubes at -20°C until DNA extraction.

Table 4.1: Plant, organic soil layer, and PHC treatment variables (n=3 for each combination).

Plant	Organic Soil Layer	PHC
Pine [P] + Lingonberry [L]	Forest floor [FH]	PHC
	Coarse woody debris [CWD]	No PHC (control)

Soil Analysis

Organic (FH and CWD) and mineral soil (Ae and Bf) samples were analyzed for total C and N content using <100-mesh samples (air-dried, then ground in a Model MM200 ball mill; Retsch, Haan, Germany) by dry combustion using a Model 1500 NC Elemental Analyzer (Fisons, Milan, Italy). The pH of organic soils was measured in a 1:2 soil to deionized H_2O suspension while 1:1 suspensions (in deionized water) were used for mineral soils (Kalra and Maynard, 1991).

LH-PCR and fragment analysis

Frozen root systems were crushed in liquid nitrogen and DNA was extracted using a CTAB (hexadecyl trimethyl ammonium bromide) protocol with an extra phenol/chloroform-isoamyl alcohol (1:1) purification step (Fujimura *et al.*, 2008). DNA extracts were further cleaned using the Wizard® PCR Preps DNA Purification System kit (Promega) to remove phenolics and other oily contaminants. These cleaned extracts were resuspended in TE buffer.

Fungal communities were characterized by amplicon length heterogeneity PCR (LH-PCR), which provides an estimate of relative abundance of genotypes in a community (Martin and Rygiewicz, 2005). For fungi, the ITS2 region of ribosomal DNA was amplified using the forward primer ITS3 (5'GCATCGATGAAGAACGCAGC) (White *et al.*, 1990) and the D3 fluorescent dye-labeled reverse primer NL4B (5'GGATTCTCACCCTCTATGAC) (Martin and Rygiewicz, 2005). ITS3 is a universal primer that binds in a conserved domain 128 bp from the 3' end of the 5.8S rDNA whereas NL4B binds in the large subunit (28S) at basidiomycete- (and ascomycete-) specific sites (Martin and Rygiewicz, 2005); PCR products are expected to be vary from approximately 400 to >600 bp in length. PCR reactions consisted of 10X PCR buffer, 2 mM dNTPs, 50 μM $MgCl_2$, 10 μM forward and reverse primers (Proligo, CO), 0.7 U Platinum Taq DNA polymerase (Invitrogen Life Technologies), and nuclease-free water (Integrated DNA Technologies, Inc.) to a final volume of 27 μL, to which 3 μL DNA (diluted 1:10) was added. The DNA Engine DYAD™ thermocycler (MJ Research, Inc., Watertown, MA) conditions were as follows: initial denaturation for 4 min at 94°C, annealing for 1 min at 48°C, and extension for 2 min, followed by 35 cycles of denaturation (94°C for 30 s), annealing (48°C for 30 s) and

extension (72°C for 1 min 30 s) and final extension at 72°C for 6 min 30 s. All PCR products were run on 1.2% agarose gels to confirm amplification.

For bacteria, the D4 fluorescent dye-labeled forward primer 27F (5′AGAGTTTGATCMTGGCTCAG) and unlabelled reverse primer 355R (5′GTCGCCTCCCGTAGGAGT) were used to amplify 16S rDNA (Mills *et al.*, 2003). PCR reactions consisted of 3 μL DNA (diluted 1:50), 10X PCR buffer, 2 mM dNTPs, 50 μM $MgCl_2$, 10 μM forward and reverse primers (Proligo, CO), 0.7 U Platinum Taq DNA polymerase (Invitrogen Life Technologies), and nuclease-free water (Integrated DNA Technologies, Inc.) to a final volume of 30 μL. The DNA Engine DYAD™ thermocycler (MJ Research, Inc., Watertown, MA) conditions were as follows: initial denaturation for 1 min at 94°C, 35 cycles of denaturation (94°C for 45 s), annealing (52°C for 45 s) and extension (72°C for 1 min 30 s), and final extension at 72°C for 10 min. All PCR products were run on 1.2% agarose gels to confirm amplification.

Fungal and bacterial samples were analyzed separately, but following the same procedure. PCR products (2 μL) were loaded into a CEQ™ 8000 sequencer (Beckman-Coulter Inc.) along with CEQ 600 bp (for fungal fragments) or CEQ 400 bp (for bacterial fragments) size standard mixture. Run conditions were 60°C separation temperature, 4 kV voltage, and 120 min separation time. Analysis was performed using the amplicon fragment length polymorphism (AFLP) program of the CEQ™ 8000 sequencer and the quartic (for fungi) or cubic (for bacteria) model for size standard with the minimum relative peak height set at 1% and a bin width of 1.5 bp.

The relative abundance of genotypes in each sample (i.e. fungal community) was calculated by relativizing the fluorescent signal strength of each fragment peak to the total peak area within each sample (Osborne *et al.*, 2006). Nonmetric Multidimensional Scaling (NMS) was calculated on the basis of a Sørensen distance measure with 50 runs with real and randomized data and a maximum of 500 iterations to assess stability (instability criterion was 0.00001) using PC-ORD 5.0 software (McCune and Mefford, 1999). A stepwise reduction in dimensionality (6D-1D) was used to minimize stress along with a random starting configuration (user-provided seeds). Stress (i.e. goodness of fit measure) and instability (i.e. measure of change in stress at each iteration) were used to evaluate the structure of the ordination results. The final solution for NMS was accepted after comparing 50 runs with real to randomized data using Monte Carlo simulations (McCune and Grace, 2002). Pairwise comparisons between groups were tested statistically with Multi-Response Permutation Procedures (MRPP), a non-parametric method that provides a statistic of the magnitude of differences between groups (i.e. effect size), given as the chance-corrected, within group agreement (A) and a p-value (McCune and Grace, 2002).

Results

Soil properties

As expected, C and N contents were much greater in organic (FH, CWD) compared to mineral (Ae, Bf) soil layers (Figure 4.1). In organic layers, mean C content in control (i.e. no PHC treatment) FH soil layers was 12.1% compared to 50.4% in CWD layers. In PHC treated soils, the amount of C increased to 21.6% in FH layers, but did not significantly change in CWD soil layers, where it was 51.6%. In Ae and Bf layers, C content did not vary in FH and CWD soil systems: levels for Ae and Bf layers were 1.6% and 1.2%, respectively, in controls compared to 3.5% and 1.5% in PHC treated systems.

N content was 0.30% in control FH layers and 0.25% in control CWD layers. In the PHC treated FH layer, %N increased to 0.45% whereas %N decreased to 0.18% in PHC treated CWD layers. N content was 0.08% and 0.07% in Ae and Bf layers, respectively; these values did not vary between soil systems or in PHC treated and control soils.

Soil pH (water) was generally lower in PHC treated compared to untreated soil systems, and was not different in FH and CWD systems. In control soil systems, pH was about 5.8 in the organic layer, 5.3 in the Ae layer, and 5.6 in the Bf layer. In PHC treated systems, pH was 5.3 in the organic layer, 5.0 in the Ae layer, and 5.6 in the Bf layer. There were no differences in soil properties at 1 and 16 weeks.

ECM morphotypes

Four months after seed germination (i.e. week 0, time of the PHC treatment), virtually all pine root tips were colonized with ECM fungi. ECM richness ranged from three to seven (mean of five) ECM morphotypes per root system. In general, ECMs, extraradical mycelia, and rhizomorphs were more extensively developed by the 16-week sampling time. The density of root tips was greater in the organic layer of the soil profile, but most root tips occurred in the Bf layer (i.e. greater relative abundance). As shown in Figure 4.1, ECMs were often associated with specific soil layers and varied in terms of relative abundance and spatial distribution on different parts of the root systems.

Eight ECM morphotypes were frequently identified on root systems, including *Cenococcum*, *MRA*, E-strain, *Amphinema*, two Russulaceae (including *Lactarius*), and two *Rhizopogon-Suillus* types. Rare ECM types were also observed, but were not included in the present analysis. Morphological descriptions of pine ECMs are provided elsewhere (Appendix A).

Clusters of *Cenococcum* ECMs dominated the upper roots of pine in the FH layer, but were never observed in the CWD layer of these double-plant systems. In PHC-treated systems, *Cenococcum* morphotypes appeared to be less abundant after 16 weeks and root tips appeared dry and less robust. *Amphinema* ECMs and external mycelia were often associated with *Cenococcum* tips near the organic-Ae layer interface, but were also occasionally observed in the Bf layer. The other ascomycetes, *MRA* and E-strain, were commonly found throughout the soil profile, but in low relative abundance.

Two members of the Russulaceae (one *Lactarius* and one *Russula* type) dominated most root systems (i.e. ECMs observed on 80-90% of root tips), particularly by the 16-week sampling time; at least one ECM was identified as a species of *Lactarius* based on the presence of laticifers. Root tips varied in colour from yellow to orange-brown and were generally unbranched and smooth or appeared velvety due to the few short unclamped hyphae that resembled cystidia early in their development. Although mainly found in the Bf layer, Russulaceae ECMs were also common on the upper lateral roots, particularly in the CWD layer.

Two *Rhizopogon-Suillus* types were found associated with root tips exclusively in the Bf layer. The first was a dark brown, coralloid morphotype. The second was white with a slightly tuberculate form. By the 16-week sampling time, both ECMs had developed extensive rhizomorph networks and hyphal fans that extended away from root tips into the Bf soil.

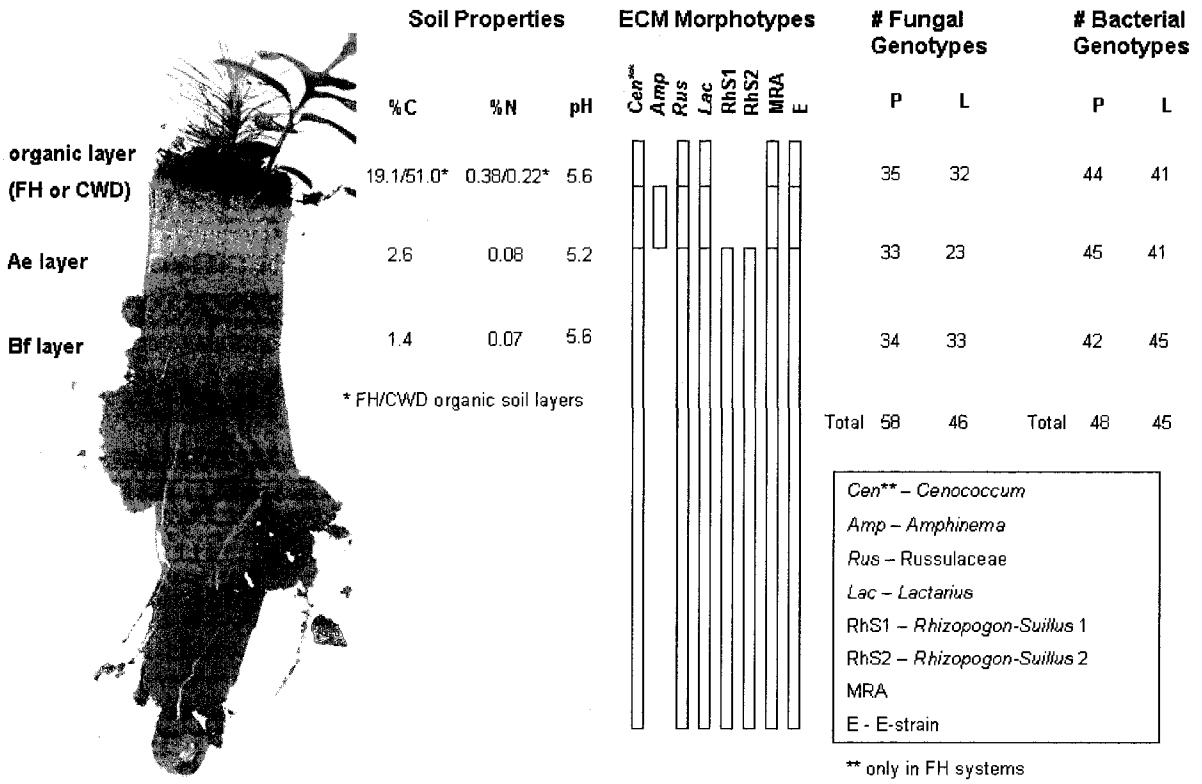

Figure 4.1: Vertical distribution of soil properties (C, N, pH), ECM morphotypes (Cen, *Cenococcum*; Amp, *Amphinema*; Lac, *Lactarius*; Rus, Russulaceae; Rh-S1, *Rhizopogon-Suillus* 1; Rh-S2, *Rhizopogon-Suillus* 2; *MRA*; E-strain), and total number of ECM/ ERM fungal and bacterial genotypes associated with pine (P) or lingonberry (L) in organic (FH or CWD), Ae and Bf soil layers of a shared-rhizosphere system.

Fungal community structure

The complete fungal dataset included 112 root samples and 75 amplicon fragments (400-690 bp in length). Multivariate analysis using nonmetric multidimensional scaling (NMS) gave a three-dimensional solution with a final stress of 18.98 and instability of 0.08. Fungal community structure varied between the 1-week and 16-week harvest times ($p<0.001$); pairwise comparisons within PHC-treated and untreated (control) systems at 1 and 16 weeks indicated that the changes in fungal community structure over time occurred in PHC-treated ($p<0.001$) systems only. PHCs showed no other effects on fungal community structure. Comparisons between fungal communities in different soil layers (organic, Ae, Bf) at 1 and 16 weeks showed significant differences ($p=0.020$) only between communities inhabiting the organic layer.

Figure 4.2 shows the NMS ordination for fungal community structure within plant and soil layer groups. Plant (i.e. pine or lingonberry) had a significant effect ($p<0.001$) on fungal community structure. Fungal communities associated with pine roots differed ($p<0.001$) from communities associated with lingonberry roots sharing the same soil system (i.e. with overlapping rhizospheres). This distinction between pine and lingonberry fungal communities is clearly visible along the 1st and 2nd ordination axes (explained 39.9% of the variation in the dataset) in Figure 4.2, where pine communities tended to group to the left, and lingonberry to the right, of the ordination space. Comparisons (using MRPP) of pine and lingonberry root communities by soil layer (organic, Ae, Bf) also revealed significant differences ($p<0.001$) in community structure for all pairwise analyses.

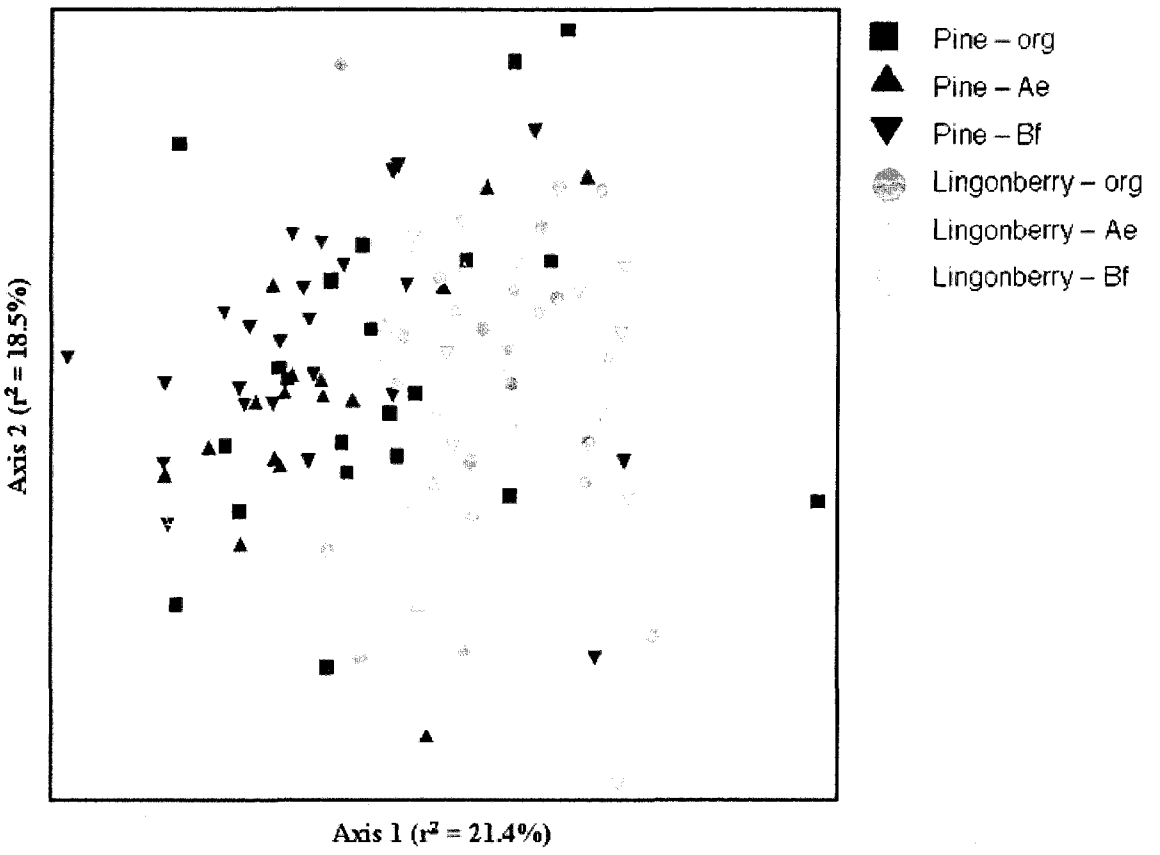

Figure 4.2: NMS ordination of fungal community structure by plant (pine or lingonberry) and organic (FH and CWD) and mineral (Ae, Bf) soil layers (stress = 18.98; instability = 0.08).

Soil properties also contributed to fungal community structure. Figure 4.3 shows the NMS ordination for fungal community structure within the three soil layers (organic, Ae, Bf) of the two soil (FH and CWD) groups along the 1st and 3rd ordination axes (explained 41.6% of the variation in the dataset). The most visible distinction appears to be between the FH and CWD systems, which tend to separate vertically. Within the layered soil systems, MRPP analysis revealed significant differences ($p=0.027$) between communities in organic and Bf layers, but not between Ae and either organic or Bf layers. Comparison of three soil layers for FH-Ae-Bf and CWD-Ae-Bf systems showed significant differences ($p<0.003$) between all combinations of the two systems, but no significant differences between layers within the same type of system (i.e. overlap of fungal community structure in all 3 layers).

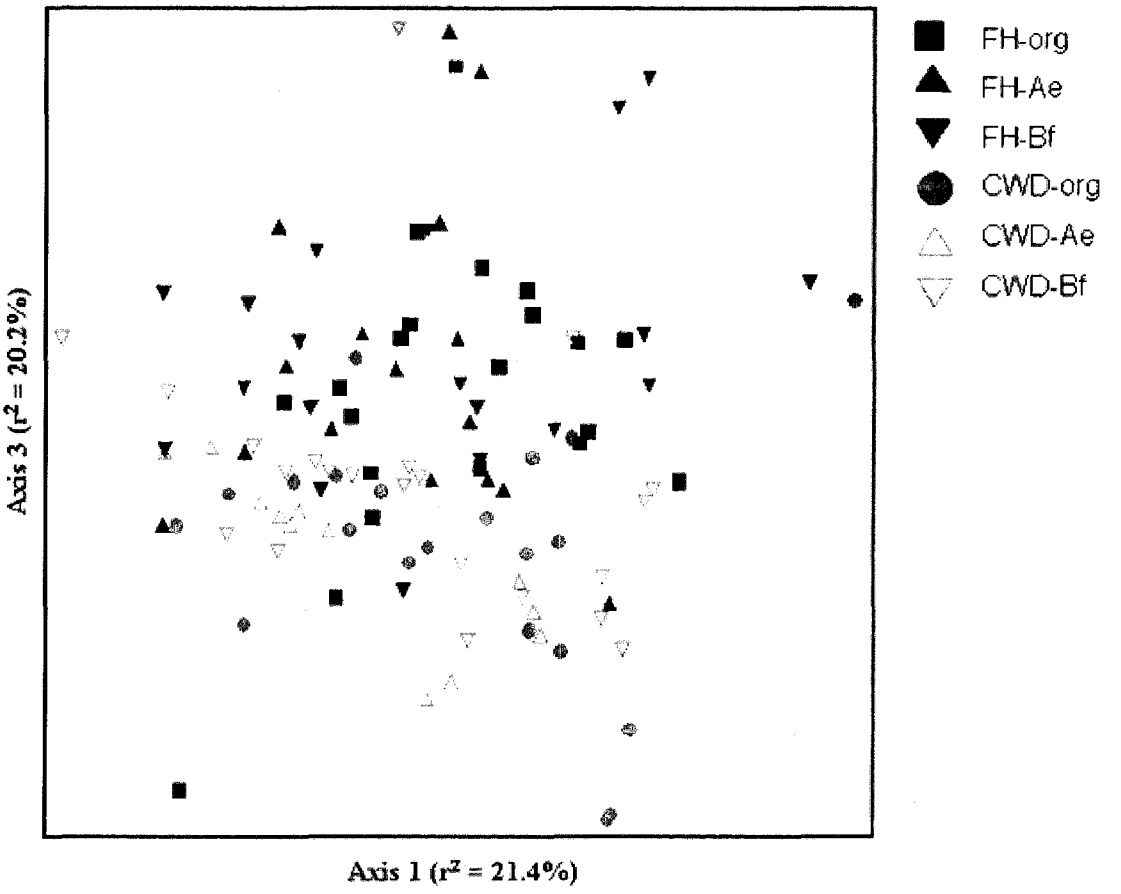

Figure 4.3: NMS ordination of fungal community structure by soil layer in the two soil systems (FH-Ae-Bf and CWD-Ae-Bf) (stress = 18.98; instability = 0.08).

Separate analyses of pine and lingonberry root systems were based on 61 root samples (58 DNA fragments ranging from 400-690 bp in length) and 51 root samples (46 DNA fragments ranging from 400-630 bp), respectively. Within plant groups, significant differences ($p=0.002$) in fungal communities were found only between the organic and Bf layers of pine (Figure 4.4a). An even closer examination of pine-fungal communities in soil layers of FH-Ae-Bf and CWD-Ae-Bf systems showed that significant differences were associated with the CWD systems and occurred between all layers: CWD-Ae ($p=0.030$), CWD-Bf ($p=0.018$), and Ae-Bf ($p=0.006$) (Figure 4.4b). Comparisons of FH-Ae-Bf systems with CWD-Ae-Bf systems showed significant differences in pine-associated fungal communities only between the Ae layers of the two systems. For lingonberry, no differences were found between soil layers, but significant differences in fungal community structure were found between all layers of FH-Ae-Bf and CWD-Ae-Bf systems (Figure 4.4).

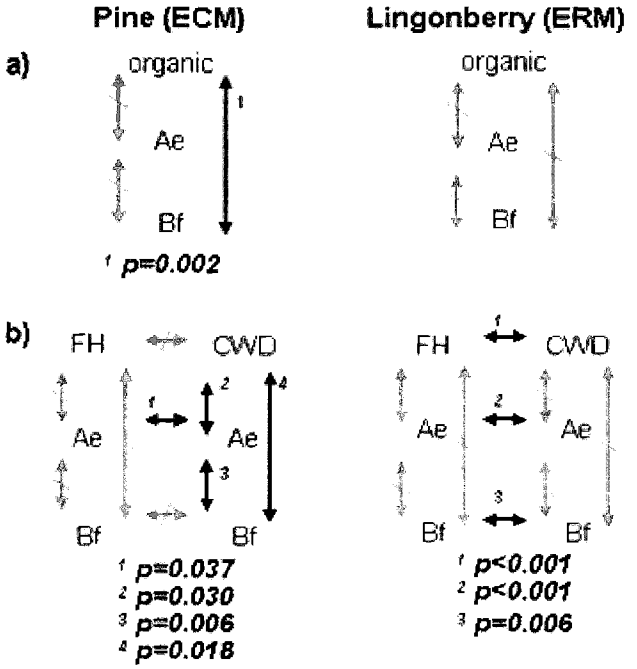

Figure 4.4: Pairwise comparisons of fungal community structure (genotypes) in pine (ECM) and lingonberry (ERM) groups: a) within soil layers; b) within and between layers of FH and CWD soil systems. Significant differences (MRPP) are represented by dark arrows and corresponding p-values; light (slashed) arrows represent no differences between groups.

Both plant systems supported the trends associated with harvest time and PHCs that were described for the complete fungal dataset. Differences in fungal community structure at 1 and 16 weeks post-PHC treatment were significant only in PHC-treated systems and there were no significant differences between PHC-treated and control communities at either 1 or 16 weeks.

Bacterial community structure

The same pine and lingonberry root systems used to assess fungal community structure were used for bacterial community structure analyses. This dataset consisted of 119 root samples and 49 DNA fragments, ranging between 300 and 420 bp. Pine roots accounted for 61 root samples (48 DNA fragments); lingonberry systems accounted for 58 root samples (45 DNA) fragments. The PHC and harvest time variables had little effect on bacterial communities, which appeared to follow the same trends in distribution as fungal communities.

NMS analysis resulted in a two-dimensional solution with a final stress of 15.96 and instability of 0.06 (Figure 4.5). As with fungi, plant (pine and lingonberry) appeared to be the dominant variable in structuring root-associated bacterial communities, but soil layer and type of soil system (i.e. FH-Ae-Bf compared to CWD-Ae-Bf) were also important variables. The figure shows clear separation of the pine from the lingonberry bacterial communities along the two ordination axes (explained 83.7% of the dataset), and some distinction between organic and mineral soil layers associated with each plant.

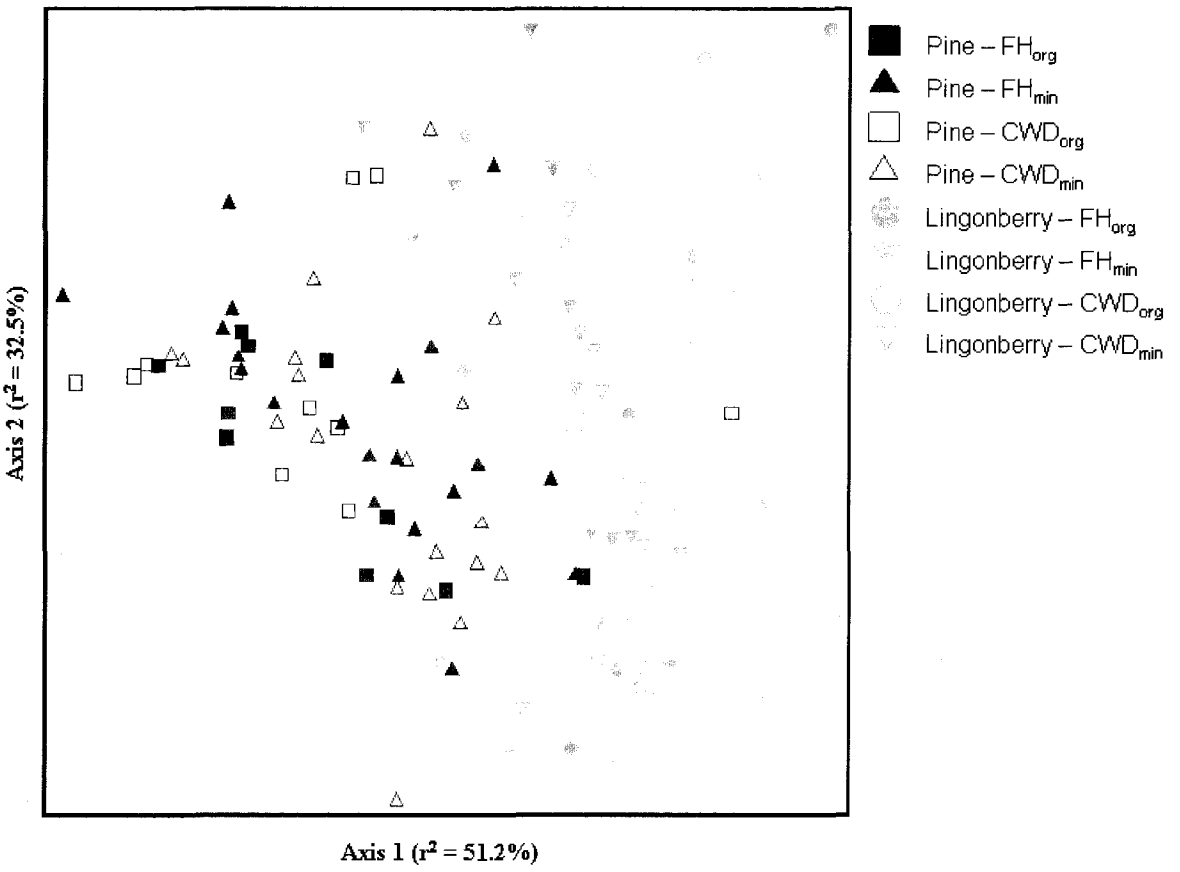

Figure 4.5: NMS of bacterial community structure associated with plant (pine or lingonberry) in organic (FH or CWD) and mineral soil layers (stress = 15.96; instability = 0.06).

Nested pairwise comparisons between bacterial communities of different plant and soil treatment groups are summarized in Figure 4.6. In combined plant groups (P+L), MRPP analysis revealed significant differences between organic and Bf layers ($p=0.005$), but not between Ae and either organic or Bf layers (i.e. same as for fungi). In separate pine and lingonberry analyses, Bf layers varied from both organic ($p<0.001$) and Ae ($p=0.005$) layers, which did not differ from each other (Figure 4.6a). Bacterial community structure varied significantly ($p<0.001$) between pine and lingonberry systems in each soil layer.

Overall, comparisons of three soil layers within FH-Ae-Bf and CWD-Ae-Bf systems showed significant differences ($p<0.001$) in bacterial community structure between all combinations of soil layers (Figure 4.6b). Within FH and CWD system types, significant differences were found between organic-Bf and Ae-Bf layers of only the FH systems ($p<0.001$ for both). Differences ($p<0.001$) in bacterial community structure in all soil layers were also observed between FH-Ae-Bf and CWD-Ae-Bf systems. Separate analyses of pine and lingonberry communities showed that the organic and Bf layers varied significantly in the CWD-Ae-Bf systems of pine ($p=0.030$) and in the FH-Ae-Bf systems of lingonberry ($p<0.001$). Pine CWD systems also differed between Ae and Bf layers ($p=0.037$). Differences ($p<0.001$) in bacterial community structure between soil systems were significant only in lingonberry systems.

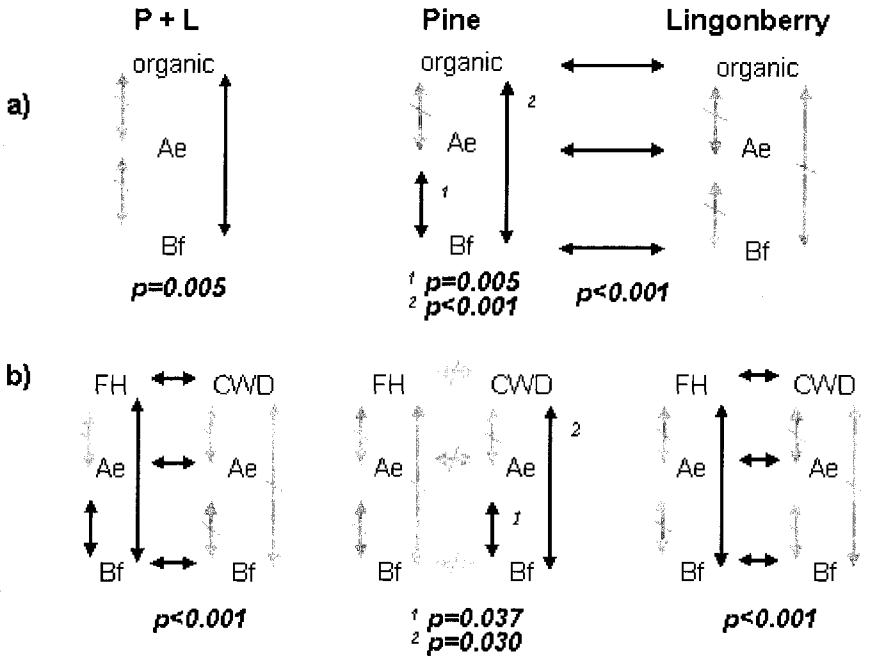

Figure 4.6: Pairwise comparisons of bacterial community structure (genotypes) in all plant systems (P+L), as well as in pine (ECM) and lingonberry (ERM) groups: a) within soil layers; b) within and between layers of FH and CWD soil systems. Significant differences (MRPP) are represented by dark arrows and corresponding p-values; light (slashed) arrows represent no differences between groups.

Discussion

This study is the first to describe the spatial distribution patterns of microbial communities from a shared rhizosphere perspective, encompassing fungal and bacterial communities associated with pine (ECM host) and lingonberry (ERM host) roots interacting within the same soils. Our results showed that plant and soil properties both contributed to community structure of root-associated communities, but that community patterns varied among the different guilds of microorganisms (i.e. ECM and ERM fungi and associated bacteria) at different spatial scales within the mycorrhizosphere. Overall, PHC contamination had little effect on the composition of root-associated microbial communities.

ECM and ERM root communities

Within our soil systems, fungal community structure differed distinctly between plants (pine and lingonberry), reflecting the specificity of host root systems to form ECM or ERM symbioses. It has long been presumed that ECM and ERM fungal communities were both spatially and functionally separate (Smith and Read, 1997). We also found that bacterial community structure varied between the different mycorrhizal root systems, indicating that differences between the ECM and ERM root environment are also important with respect to bacterial niche differentiation. Mycorrhizal interactions with heterotrophic bacterial communities associated with the mantle and extraradical mycelia of ECM fungi and ERM roots are important for mobilization, uptake and translocation of nutrients required for fungal and, ultimately, plant growth (Burke and Cairney, 1998). As the establishment of ECM symbiosis may alter root morphology (mantle and extraradical mycelia) and physiology (e.g. membrane permeability, exudation patterns) in different ways than ERM symbiosis,

particularly with respect to quality and quantity of root exudates (Rygiewicz and Anderson, 1994), it is not surprising that mycorrhizal root-influenced differences in habitat supported distinct bacterial communities.

High genotype richness (i.e. 58 and 46 DNA fragments, respectively for ECM and ERM fungi, and 49 DNA fragments for root-associated bacteria) was observed for all three guilds of microorganisms. Although high richness has been related to ecological resiliency, there is no current sense of this threshold, particularly with respect to molecular assessment methods. For ECMs, genotype richness was much greater than morphotype richness, which consisted of eight common (and several rare) morphotypes. Discrepancies between morphological and molecular analyses of ECMs have been reported in many studies and reflect the dynamic character of mycorrhizal systems (Rosling *et al.*, 2003). High genotype richness could be due to the presence of inconspicuous or rare ECM fungi or other root-associated fungi such as ascomycetous double colonizers (Rosling *et al.*, 2003), saprotrophs (Lindahl *et al.*, 2007) or dark septate endophytes (Mandyam and Jumpponen, 2005) that may coexist with ECM fungi on the roots. Their DNA was likely amplified along with the ECM fungal DNA extracted from the washed root systems and included in the total genotype count following LH-PCR. Secondary colonization (i.e. succession) of root tips already colonized by ECM fungi also may have occurred; we noted a trend of increasing richness of ECM morphotypes and genotypes over the 16 weeks of the study that was generally taken as a reflection of community development with time on the young root systems. Succession in ECM root colonization was also reported by Massicotte *et al.* (1999). In addition, an enhancement of genotype richness could result from intraspecific variation within fungal taxa, which could

increase the number of DNA fragments based on variables characters of ITS rDNA that are unrelated to functional diversity (Horton, 2002). The same factors that contributed to genotype richness in ECM systems also likely contributed to enhanced richness in ERM systems.

We also found substantial overlap in fragment lengths (genotypes) occurring in pine (ECM) and lingonberry (ERM) communities, both for fungi and bacteria. For fungi, this could be due to similarities in communities of non-mycorrhizal fungi (i.e. saprotrophs, dark septates, etc.) associated with pine and lingonberry roots in the shared rhizosphere. These non-mycorrhizal fungi may not express host specificity to the same extent as ECM and ERM fungi; communities may be structured more by the quantity rather than quality of available substrates (i.e. exudates in the rhizosphere). Alternatively, the presence of shared fungal genotypes in the two plant systems could be simply coincidental (i.e. as fragment lengths do not reflect taxa in LH-PCR), or may indicate sharing of mycorrhizal symbionts between root systems. In resynthesis experiments, Vrålstad *et al.* (2002) showed that several strains of ERM fungi comprising the *Rhizoscyphus ericae* aggregate formed true ECMs with conifer (spruce and pine) and angiosperm (birch) species, although no isolates formed both ECMs and ERMs. Villarreal-Ruiz *et al.* (2004) reported the ability of a fungus from the *R. ericae* aggregate to form simultaneously both ECMs and ERMs in culture with *Pinus sylvestris* and *Vaccinium myrtillus* seedlings; however, any sharing of mycorrhizal symbionts has yet to be demonstrated in soil systems. Although we observed close interactions between pine and lingonberry roots in the soils in this study, the resolution of the community fingerprinting

method (i.e. LH-PCR) was not sufficient to demonstrate sharing of mycorrhizal fungal symbionts (i.e. only sharing of amplicon fragment lengths).

In studies relating microbial communities to ecological processes at larger spatial scales (e.g. rhizosphere, stand, etc.), an understanding of spatial patterns of functional guilds of microorganisms may be more important than the taxonomical details. Community fingerprinting methods such as LH-PCR and terminal restriction fragment length polymorphism (TRFLP) provide little taxonomic resolution of microbial communities compared to methods such as sequencing, but are expected to provide sufficient resolution to separate communities based on broad variables (Kuyper and Landeweert, 2002). Mills *et al.* (2003) found no difference in resolution between LH-PCR and TRFLP in assessments of PHC-degrading bacterial communities. The coarse filter approach used here to describe patterns of root-associated communities in the shared rhizosphere potentially offers ecologically relevant information at the expense of some fine-scale resolution.

Soil properties

Soil properties of the reconstructed systems used in this study generally resembled soil properties at the field site. Exceptions included a slightly lower C:N ratio (about 40) in the FH layer of the bioassay and less acidity in all three layers compared to the forest site. PHC contamination altered soil properties by increasing C content, particularly in the organic (FH) soil layers, and generally lowering pH in organic and Ae layers. Lower pH could be partly due to production of metabolic acids and may reflect enhanced microbial activity in response to addition of readily metabolizable C substrates present in PHCs. Lindahl *et al.* (2007)

found increased saprotroph activity in the vicinity of mycorrhizal root tips with increased C availability. Nitrogen levels increased in FH layers, decreased in CWD layers, but remained the same (i.e. much lower than organic soil) in both mineral soil layers. Due to the high porosity in organic soil layers, PHCs, particularly more hydrophobic compounds, tend to be retained in these layers as compared to mineral soils. Hydrophobic compounds also have a greater tendency to adsorb to organic soil components than to mineral ones (Xing *et al.*, 1994). Thus, microbial communities in organic layers must inhabit PHC-contaminated habitat to a greater extent than communities inhabiting the mineral layers below, which consisted mainly of sand particles with few binding sites for the PHC chemicals that eventually made their way down the profile along the roots.

However, changes to soil properties related to PHC contamination appeared to have little effect on community composition of root-associated microorganisms (fungi and bacteria), at least within the 16-week duration of the experiment. Setälä *et al.* (2000) found that soil microbial communities appeared to exhibit high resilience to environmental disturbances when soil organic layers (e.g. humus, woody debris, etc.) were not severely disrupted. The mycorrhizosphere may offer some physical protection from potential toxic effects (e.g. solvent shock) of PHC treatment, surfaces for biofilm formation (themselves, protective structures), as well as a steady supply of C substrates supporting ongoing microbial metabolism. The extent to which these protective factors extend into the rhizosphere remains unknown and was not investigated in this study.

In the shared rhizosphere systems studied here, soil properties exerted two kinds of effects on microbial community structure: contrasting properties of the FH-Ae-Bf and CWD-Ae-Bf soil systems, which are potentially important during (mycor)rhizosphere development, and vertical segregation in the soil profile (reflection of broad changes in physical soil properties).

FH and CWD systems

The planting of pine seeds into FH and CWD organic layers provided opportunities for potentially different indigenous microorganisms to colonize the newly germinating roots in these two systems. The greater C:N ratio of the CWD layer could potentially enhance different communities of ECM fungi as well as saprotrophic communities. However, differences between root-associated communities (ECM fungi and bacteria) inhabiting the different soil layers were generally not observed in pine systems, except in Ae layer comparisons. This difference may be due to percolation of different metabolic products from CWD decomposition (i.e. compared to FH) downward in the soil profile, which could influence microbial communities in the Ae layer below.

Lingonberry communities appeared to vary more between soil systems (i.e. FH-Ae-Bf and CWD-Ae-Bf) than between soil layers; the same trend was observed for both fungal and bacterial communities associated with lingonberry roots. It is possible that the soil habitat in and below CWD was different in ways (that we did not measure) from FH systems that supported different groups of microorganisms associated with lingonberry roots. Unlike pine, lingonberry cuttings (rooted in reconstructed forest soils) were transplanted into the soil

systems, which may have influenced fungal and bacterial colonization in unknown ways (e.g. introduced some competition). Further interpretation of this finding is difficult in light of the current limited understanding of ERM fungi.

Vertical segregation

ECM fungal communities exhibited vertical community structure, reflecting the direct interactions between fungi and soil in the ectomycorrhizal association. In general, ECM fungal communities in organic soil layers were distinctly different from Bf-layer, but not Ae-layer, communities. In our soil systems, the boundaries above and below the Ae layer were not well defined and ECM fungal communities often overlapped in these zones. Rosling *et al.* (2003) found that ECM community composition varied between organic, eluvial and deeper mineral horizons of a Swedish podzol, and that most taxa described from the organic layer were also found in the eluvial horizon below. Genney *et al.* (2006) found that the layered nature of the soil substrate was more likely to encourage horizontal rather than vertical extension of mycelia and that ECMs and extraradical mycelia often occupied adjacent layers of organic and upper mineral soils. A comparison of FH and CWD soil systems revealed that differences between soil layers that influenced community structure were mainly attributable to CWD systems. The overlap in fungal communities between layers again reflects the continuity and dynamics of ECM systems.

In contrast, ERM fungal communities of lingonberry did not vary in the different soil layers. Due to their endomycorrhizal nature, ERM fungal communities may be less influenced by differences in soil properties compared to ECM fungi that are in direct contact with soil on

the outer root surfaces and extraradical mycelia. In addition, compared to ECM fungi, there are relatively few ERM fungi, which may occupy fewer niches. As epidermal root cells appear to be composite structures that are colonized by a variety of fungal symbionts, each cell potentially functions as a separate unit (Berch *et al.*, 2002; Perotto *et al.*, 2002). Thus, variation in ERM community structure may occur at the level of individual hair roots rather than the level of the whole root system (i.e. rhizosphere), as for ECM communities.

Bacterial communities that inhabit the outer surfaces of the ECM mantle and mycelia, as well as the ERM roots, also directly interact with the soil environment, and were found to exhibit complex patterns of vertical segregation similar to ECM fungal community patterns. Depending on the resolution of analysis, bacterial community structure was found to vary between Bf and organic and Bf and Ae layers. In pine systems, differences were associated with CWD systems, whereas in lingonberry systems, differences were associated with FH systems. Changes in soil chemical, mineralogical, and structural properties with depth create complex and variable sets of microbial habitats over very small distances, to which free-living bacterial cells may be particularly sensitive (Genney *et al.*, 2006). Due to their small size and structural vulnerability to environmental stresses, bacterial communities may be expected to vary along much smaller spatial scales compared to the fungal communities with which they are associated.

The vertical distribution patterns of ECM morphotypes described on the root systems of pine seedlings in this study were consistent with those described from field-based studies in European forests (Rosling *et al.*, 2003; Tedersoo *et al.*, 2003; Baier *et al.*, 2006; Genney *et*

al., 2006; Lindahl *et al.*, 2007). In our soil systems, the density of ECM root tips was greater in organic compared to mineral soil layers, but this was not related to either morphotype or genotype richness (genotype richness was also not different between soil layers for ERM fungi and bacteria). Most of the root system (i.e. much higher abundance of mycorrhizal roots) occurred in mineral (i.e. Bf layer) soil layers. Some ECMs (*Cenococcum*, *Amphinema*, Russulaceae and *Rhizopogon-Suillus* types) dominated certain parts of the soil profile, while other ECMs (MRA and E-strain) occurred throughout the profile, usually at low relative abundance. These community patterns are not always revealed from studies using soil cores where a fairly arbitrary and shallow sample of the belowground habitat is taken. Our rhizosphere approach had the advantage of sampling entire root systems (albeit small and young), which represent ecological units on the landscape. For seedlings, at least, this approach provided a more precise picture of the abundance component of diversity, which may be important in terms of ecological functions.

Similar ECM partitioning patterns in temperate forests (USA) were attributed to ECM specificity (or generality) for resource use in the different soil layers (Dickie *et al.*, 2002). For example, soil organic matter (SOM) is an important source of substrates for decomposition and nutrient cycling in forest ecosystems (Read and Perez-Moreno, 2003). The prevalence of ECM fungi in well-degraded litter and humus layers of organic soils supports the hypothesis that they play a significant role in mobilizing N from the more recalcitrant organic matter in boreal forest soils (Lindahl *et al.*, 2007). Linkages between ECM community structure and function have been inferred using ECM exploration types (i.e. groups of ECM morphotypes defined by the amount, organization and extent of the

extraradical mycelia) as the units for analysis (Agerer, 2001). Patterns are thought to indicate differential resource utilization, which suggests that ECMs have distinct foraging strategies and different capacities for resource acquisition (Agerer, 2001; Baier *et al.*, 2006). The relative abundance of hyphae of various ECMs may strongly influence host and ecosystem function (Dickie *et al.*, 2002).

In our study, forest floor (FH) layers were dominated by *Cenococcum*, which has often been associated with high humus content and a large C:N ratio (Rosling *et al.*, 2003; Baier *et al.*, 2006). *Cenococcum* is a short distance exploration type (Agerer, 2001) characterized by a dense cover of emanating hyphae, suitable for making multiple contacts with the loose organic cover of the forest floor (Baier *et al.*, 2006). Although *Cenococcum* tends to be tolerant of changing environmental conditions (e.g. temperature, moisture) typical of organic soil layers in forests, some morphotypes in the current study appeared dry, less robust, and less abundant after 16 weeks in the PHC treated systems compared to untreated controls (Chapter 2). *Cenococcum* is often associated with *Amphinema*, which tends to inhabit the more environmentally stable interface between the organic and Ae layers (Rosling *et al.*, 2003; Baier *et al.*, 2006). *Amphinema* (as well as *Piloderma*, which did not colonize root tips in this study, but whose distinctive yellow hyphae were frequently observed in organic soil layers) has been described as medium-distance (fringe) exploration type characterized by fans of emanating hyphae with extended contact to the soil, as well as rhizomorphs that ramify and interconnect repeatedly (Agerer, 2001).

Whereas *Cenococcum* dominated the FH layer, it was consistently absent from the CWD layer, which was characterized by a greater C:N ratio due to the prevalence of lignified substrates as well as other physical differences. Instead, the two common Russulaceae morphotypes tended to dominate root systems in CWD (dead wood <2.5 cm diameter). *Lactarius* (and other Russulaceae) ECMs are typically associated with acidic, low-N soils, and may also contribute directly to decomposition via oxidative enzyme activity (Lilleskov *et al.*, 2002). CWD is also an important habitat for resupinate thelephoroid and athelioid fungi, as well as Sebacinaceae (Tedersoo *et al.*, 2003). We found that thelephoroid ECMs represented a rare component of the ECM fungi described in this study; these fungi tend to be inconspicuous (and also may not establish with young seedlings), but they likely contributed to genotype richness from roots in CWD.

Pine roots in mineral soils were frequently dominated by the Russulaceae ECMs, which often accounted for more than three quarters of the total number of roots tips per seedling. These contact exploration types had smooth mantles with few emanating hyphae and were well equipped to explore dense soil horizons with narrow pores (Agerer, 2001; Baier *et al.*, 2006). They also tended to have preference for higher bulk density of mineral compared to organic soil layers (Genney *et al.*, 2006).

The two *Rhizopogon-Suillus* ECMs described in this study were exclusively found in the Bf layer. Though not usually dominant on individual root systems, extensive extraradical mycelia and rhizomorphs of these ECMs likely accounted for a high proportion of biomass. *Suillus* and *Paxillus* morphotypes have been studied extensively in the lab (including with

respect to PHC biodegradation), but they have not been frequently described from field studies (Genney *et al.*, 2006). The lack of field observations may be an artifact of the tendency to focus studies on upper soil layers only, missing yet another functional component of the system; Rosling *et al.* (2003) found *Suillus luteus* in mineral layers in 2 (of 3) 52-cm columns of soil. Both *Suillus* and *Rhizopogon* have long-distance exploration strategies and produce highly visible thick rhizomorphs from tuberculate ECM tips (Agerer, 2001). Proliferation of hyphal fans towards suitable metabolic substrates in microcosm soils has been reported by Bending and Read (1997); in this study, we frequently observed hyphal fans extending into both PHC treated and control mineral soils. Fungal species present in small numbers as mycorrhizas but that form extensive extraradical mycelia may be functionally very important for C and N cycling (Genney *et al.*, 2006).

Conclusions

Mycorrhizas, along with the heterotrophic microorganisms tightly associated with the mycorrhizosphere, occupy the structural and functional interface for carbon and nutrient cycling between the aboveground and belowground food webs in northern forests. Interruptions in community functions through environmental disturbance such as PHC contamination could potentially disrupt essential ecosystem processes at a landscape scale. For the guilds of microorganisms at this trophic level (i.e. ECM fungi, ERM fungi and bacteria), PHCs did not appear to exert many detrimental impacts. However, it is unknown whether ongoing community development will occur along the same trajectory over the longer term in PHC contaminated systems compared to uncontaminated systems or whether this is functionally meaningful in terms of ecosystem processes. Furthermore, detrimental

impacts to microbial groups that are spatially abundant or possess more specialized functions could lead to community changes over time, so longer term studies are warranted. Analysis of variation in community structure at the rhizosphere scale gives a broad sense of community function by relating changes to habitat properties. Our finding that community patterns varied with both plant and soil characteristics and at different scales for the three groups assessed contributes to the greater understanding of forest soil ecology and sustainability.

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Chapter 5: Conclusions and future considerations for microbial ecology and sustainability management in PHC-contaminated northern forest ecosystems

This research was based on the premise that oil spills occur in boreal forest ecosystems with unknown scope and frequency. In addition, the fate and ecological impacts of petroleum hydrocarbon (PHC) contaminants in forest soils are not well understood from either eco-toxicological or microbial ecological perspectives. Mycorrhizal systems (i.e. plant-fungal symbioses and associated heterotrophic bacterial communities) represent the dominant microbial biomass in northern forest soils and may be most vulnerable to the environmental impacts of direct PHC exposure. Conservation of the integrity of mycorrhizal systems in contaminated forests may be key for management for both ecological resilience and remediation in a sustainability context.

The purpose of this research was to examine interactions between PHCs and mycorrhizal communities in the rhizosphere, where different guilds of microorganisms interact within the larger trophic group tightly linked to decomposition, carbon and nutrient cycling, and primary production. This work was both experimental and exploratory in nature, as hypotheses were both tested and generated. Several methodological approaches were simultaneously used to gain a better understanding of the physical, chemical and biological changes that occurred in the rhizosphere of sub-boreal forest soils following contamination with ecologically relevant (i.e. equivalent to several tonnes per hectare) levels of oil. Insights along a number of themes are presented in the following paragraphs.

Rhizosphere model for mycorrhizal systems

Our model system was comprised of the root system of a single plant (pine, birch or lingonberry), germinated (or transplanted, in the case of lingonberry) into vertically-structured, field-collected, sub-boreal forest soils. These plant-soil systems attempted to incorporate some of the complexities of soil and microbial community structure in the rhizosphere, but within the confines of the bioassay (i.e. limited dispersal, etc.). These systems captured a sub-set of the soil microbial community diversity expected *in situ*, which included ecto- (ECM) and ericoid (ERM) mycorrhizal fungal propagules capable of forming symbioses with different root systems in different soil types, under greenhouse conditions. Incorporation of soil vertical structure and depth expanded microbial habitat and niche, and revealed features of ectomycorrhizal (ECM) fungal diversity (e.g. extensive extraradical mycelia of the relatively abundant *Rhizopogon-Suillus* 1 morphotype) that have generally been overlooked in the deeper mineral soils.

The rhizosphere represents a discrete functional unit that repeats over northern forest landscapes. A single rhizosphere obviously does not represent the full complexity of the forest soil environment, where dynamic interactions between many different types of rhizospheres exhibit emergent properties (i.e. the community possesses capabilities that its individuals lack) (Allen *et al.*, 2003). However, concurrent assessment of plant, soil and microbial community properties at the rhizosphere level had the advantage of allowing some extrapolation to broad spatial scales over landscapes exhibiting high levels of environmental heterogeneity, a necessity for application to sustainable land management. At the same time, details of microbial communities at greater levels of taxonomic, genetic, or physiological

resolution could be assessed at the level of the individual root tip. Thus, the rhizosphere scale is intermediate to species-based approaches for understanding functional diversity and ecophysiology on smaller scales, and approaches for assessing spatial distribution and ecological functions on larger scales. In this baseline study, we focused on structure, distribution, and broad ecological functions of microbial communities at the rhizosphere scale.

A characteristic of more ecologically inclusive studies is greater variation within treatment groups, which can impair the ability to detect significant differences between them. Here, we used several methodological approaches and multivariate statistical analyses to assess changes in different system components and to look for consistent trends potentially pointing to biological significance. The remaining unexplained variation is likely the result of noise from unmeasured environmental and spatial variability (Ramette and Tiedje, 2007).

Ecological integrity and resilience

Environmental disturbance provides a means for understanding forest stability/ resilience arising from complex interactions within ecosystems (Amaranthus, 1998). We assessed environmental changes associated with soil fertility and physical structure that were expected to impact community structure and function of microorganisms and higher trophic-level organisms. In our systems, surface contamination with crude oil led to increased concentrations of potentially toxic PHC chemicals ranging from <nC10 to >nC50 in size (GC-FID analysis). Soil C analysis confirmed that C content increased in PHC treated compared to untreated (control) soils, whereas N content remained unchanged. Thus, PHCs

may potentially inhibit some microorganisms (due to toxicity) and stimulate others (due to C availability). The more acidic pH that was generally associated with PHC treated soils supports the hypothesis that microbial activity was enhanced, although this was not explicitly tested. Although we found no differences in community structure (based on relative abundance of microbial components, not just richness) between PHC treated and control systems, this may be a reflection of the relatively low-resolution method used (i.e. LH-PCR), relative simplicity of the plant-soil (rhizosphere) systems, and relatively short period of study (16 weeks). Clearly there is a need for future field-based studies incorporating older and deeper root systems, different types of plant hosts and soil types, and on greater scales of space and time to more fully understand these interactions.

Mycorrhizal communities were generally resilient to ecologically relevant levels of PHC contamination (i.e. equivalent to ~7-22 tonnes per hectare, which is within the potential range of contamination levels expected from a burst pipeline, etc.) when the integrity of the system was conserved (i.e. no further disturbance to mycorrhizal roots or soil structure). In intact systems, most PHCs were retained in the organic soil layers (GC-FID analysis), which may have limited PHC exposure of mycorrhizas and associated heterotrophic communities at greater depths in the soil profile. Although microbial communities inhabiting the organic layers were exposed to far greater concentrations of PHCs than deeper communities, morphological examinations of individual ECMs and community fingerprinting (LH-PCR) studies revealed few changes in community structure (based on relative abundance of the fungal and bacterial components of each rhizosphere community) attributable to PHC contamination. We hypothesize that the mycorrhizosphere may also have served a protective

function for both mycorrhizal fungi and associated heterotrophic communities, possibly through exclusion of potentially toxic PHCs (i.e. due to properties of fungal cell walls, biofilm formation, and solvent flow along the mycelial surface). However, it was difficult to separate the effects of chemical toxicity from habitat changes (e.g. altered carbon/ nutrient supply, pH, water holding capacity, etc.) in PHC-contaminated soil systems.

Morphological and DNA-based analyses do not directly address questions concerning PHC impacts on ecological functions because they reveal little of what the organisms are doing. Using biochemical (enzyme-based) methods to assess C metabolism, we found that PHC treatment had no effect on ECM laccase activity (for catalyzing the opening of aromatic ring structures) or on bacterial community C use profiles for substrates commonly found in the rhizosphere. More detailed functional analyses are needed to better understand relationships between PHC contamination and microbial functions, but these coarse-filter findings also point to general system resilience.

PHC biodegradation and functional redundancy

PHC concentrations (GC-FID analysis) generally decreased in all plant-soil systems over 16 weeks, indicating an inherent capacity for PHC biodegradation. Biodegradation was particularly evident in pine and birch (ECM) systems that were grown in soils with FH and CWD organic layers providing the initial microbial inoculum. Both the forest floor (FH) and coarse woody debris (CWD) organic layers were expected to contain microorganisms well adapted for decomposition/ biodegradation of C substrates typically found in these soils. The structure of ECM fungal and bacterial communities varied between these plant-soil systems,

indicating functional redundancy for PHC biodegradation. Standing *et al.* (2007) suggested that functional redundancy among widespread soil processes such as decomposition/ biodegradation may be so large that relationships between microbial diversity and ecosystem function may lose relevance at larger spatial scales. Our results indicated redundancy for C substrate metabolism for individual ECMs (laccase assay) as well as for bacterial communities (CLPP analysis). Insights into functional heterogeneity in biodegradation processes (i.e. specific metabolic reactions along catabolic pathways) within these communities require higher levels of resolution than offered in this study and should be the focus of future research.

ECMs appear to enhance biodegradation via at least two mechanisms. Our results showed that greater densities of microorganisms capable of PHC biodegradation were supported in the mycorrhizosphere compared to non-rhizosphere soil (i.e. mycorrhizosphere effect). This large heterotrophic biomass is likely fueled through addition of reduced C substrates in PHC mixtures, resulting in a generally stimulatory effect on biodegradation. In unplanted soils, we found greater bacterial densities in PHC-treated compared to untreated (control) soils, indicating that availability of readily metabolizable C substrates is at least one factor influencing the density of microbial communities in the mycorrhizosphere. In addition, we observed that the ECM extraradical mycelia greatly expanded the surface area for potential bacterial colonization and biofilm formation, which could also enhance PHC biodegradation through expansion of habitat and niche of biodegrading communities.

In the absence of mycorrhizosphere (i.e. in unplanted systems), levels of the smaller (<nC16) PHC chemicals were significantly reduced by indigenous bacterial communities, but levels of the larger (nC16+) PHCs did not change substantially. This provides indirect evidence for a stimulatory role of ECMs in the biodegradation process. More direct evidence was inferred from our finding that many ECMs exhibited the potential to open aromatic rings via laccase secretion, thus contributing to the metabolic synergy for PHC biodegradation that appears to exist within the mycorrhizosphere community. Laccase secretion by ECMs is one of many potential functions that may impact PHC biodegradation in forest soils for which we have no clear understanding with respect to ecological significance. Many of the dominant ECMs (e.g. *Rhizopogon-Suillus* 1, Russulaceae 1, and *Lactarius*) described in our study exhibited high laccase activity, both at the root tip and along the extraradical mycelia/ rhizomorphs. These morphotypes tended to inhabit the deeper, mineral soil layers and have not been well studied. The dominant ECMs may represent the most functionally relevant community component (i.e. greater biomass and greater contribution to nutrient uptake and C demand), with the numerous minor (less frequently occurring) ECMs representing functional equivalents of the dominant types, but with different environmental requirements and tolerances (Allen *et al.*, 2003). Thus, the minor types may contribute to ecosystem resilience in changing environmental conditions (i.e. disturbance) that are capable of replacing the dominant ECMs should they decline (i.e. insurance effect). This hypothesis remains to be specifically tested for mycorrhizal fungal communities, particularly with respect to contaminated soil management.

We did not find significant PHC biodegradation in the systems where additional complexity was incorporated (i.e. double-plant systems with ECM and ERM hosts or FHoil soil systems that contained residual PHCs from the previous contamination event) into the experimental design. The additional complexity may have overwhelmed the spatial and temporal limitations of the bioassay, resulting in greater within-group variation. In the double-plant systems, we found greater diversity (richness and relative abundance) of fungal and bacterial communities than in single-plant systems, and each root system supported both shared and distinct components of microbial communities. We hypothesized that double-plant systems augmented the level of competitive interactions (perhaps by providing more specialized niches owing to properties of the different root systems) compared to single-plant systems, which may have inhibited the overall biodegradation process at key steps along the biodegradation pathway. In the FHoil soils, residual PHCs probably consisted of a greater proportion of larger (>C16) PHCs that may be degraded more slowly and be less available to microbial metabolic systems. In terms of biodegradation potential based on community profiles, FHoil soil system fungal and bacterial communities varied no more from FH and CWD systems than these latter systems varied from one another.

Plants and soils

Soil microbial communities exhibit clear spatial (and temporal) patterns across landscapes based on environmental heterogeneity (niche) and the legacy of past disturbance events (Ramette and Tiedje, 2007). In this study, we found that the plant was the primary determinant of mycorrhizal fungal and associated bacterial community structure. Both morphological examinations of individual roots and community fingerprinting (LH-PCR)

studies revealed distinct fungal communities for pine, birch and lingonberry systems. This reflects the specificity of host root systems to form ECM or ERM symbioses, which were expected to be both spatially and functionally separate. This distinction between ECM and ERM fungal communities was maintained in the double-plant systems where the pine and lingonberry root systems were in close proximity. In addition, ECM fungal communities exhibited specificities for either the coniferous (pine) or deciduous (birch) host, which may have been due to differences in root structure or exudation patterns. Surprisingly, bacterial community structure also varied between the different mycorrhizal root systems, indicating that differences between ECM and ERM root environments are also important with respect to bacterial niche differentiation. Differences in microbial community structure between plant systems reflect the many potential plant-fungal combinations that occur in repeatable units across landscapes (Allen *et al.*, 2003). How the diversity of the plant community impacts microbial community structure, ecosystem resilience and biodegradation capacity following PHC contamination is an important consideration in both ecological and management contexts.

Differing properties of the soil environment exerted two kinds of effects on microbial community structure. First, communities (morphology and LH-PCR) varied between the three soil systems tested, which were characterized by different organic layers (FH, CWD, and FHoil) providing the initial microbial inoculum during early plant growth and (mycor)rhizosphere development. Second, communities exhibited vertical segregation in the soil profile (organic, Ae, and Bf layers), which is a reflection of broad changes in physical and chemical soil properties. Results from the shared rhizosphere study (i.e. double-plant

systems with both pine and lingonberry) showed that community patterns varied among the different guilds of microorganisms (i.e. ECM and ERM fungi and associated bacteria) at different spatial scales within the mycorrhizosphere and were related to certain environmental variables. ECM fungal communities, which are in direct contact with soil on the outer root surfaces and extraradical mycelia, were found to vary between different soil layers within systems, as well as between the three organic soil layers of different systems. In contrast, ERM communities appeared to be less influenced by vertical differences in soil properties, but to depend more on properties of the different organic soil systems, which may influence the initial microbial inoculum composition. Variation in ERM community structure may occur at the level of individual hair roots consisting of epidermal cells potentially functioning as separate units rather than the level of the whole root system (i.e. rhizosphere), as for ECM communities. Bacterial communities varied both within and between soil systems. Due to their small size and structural vulnerability to environmental stresses, bacterial communities may be expected to vary along much smaller spatial scales compared to the fungal communities with which they are associated. These findings help form the base for a better understanding of connections, linkages, and scale-related relevance of mycorrhizosphere/ ecosystem components that contribute to ecological processes at landscape scales and to predict how ecosystems may respond to environmental disturbances such as soil contamination. However, the identification of common patterns of biotic and environmental variation remains a central issue in microbial ecology.

Management in PHC contaminated forests

Currently, there is little ecological foundation for remediation strategies in PHC-contaminated forest soils. Rather, remediation depends on contaminant (e.g. PAH) levels and perceived toxicological threats to soil quality, forest productivity, and wildlife, intertwined with the economic incentive of recovering spilled oil. There is no evidence that a single spill event in a forest poses significant ecological threats; studies suggest that over time scales of several years, many PHCs are biodegraded via central metabolic pathways of the indigenous soil microbiota or are transformed via specific enzymatic pathways to products that are incorporated into the soil organic matter. Our results suggest that surface application of relatively high rates of crude oil to plant-soil systems had little negative impact on mycorrhizal fungal or bacterial communities in the rhizosphere, and also showed that significant PHC biodegradation occurred within 16 weeks of treatment.

Sustainable management strategies for northern forests need to preserve diverse environmental, social, and economic values for large areas over long periods of time. Mycorrhizal systems consist of functional groups of soil biota that underpin ecosystem services such as decomposition, biogeochemical cycling, primary production, soil stability, and C sequestration, to name a few (Barrios, 2007). Thus, maintenance of mycorrhizal integrity, which includes both the plant community and soil environment, should be an objective of remediation strategies for PHC contaminated soils. Ideally, remediation should utilize knowledge of the microbial communities present in contaminated soil environments, their metabolic abilities, and their likely response to changes in environmental conditions.

In situ bioremediation is considered a low-disturbance and cost-effective strategy for managing contaminated forest soils on large scales. Our results support the hypothesis that the intrinsic capacity for biodegradation is maintained in PHC contaminated soils in the absence of further environmental disturbance. This intrinsic biodegradation could be enhanced through various augmentations to the process. For example, addition of organic or woody material to the forest floor may extend the habitat of mycorrhizal systems (i.e. increase mycorrhizosphere volume) and provide energy-rich substrates for co-metabolism of the more recalcitrant PHC chemicals. Addition of N fertilizer has been used to relieve soils of the N deficiency that is often associated with PHC contamination (not found in our study); however, fertilization may also lead to conditions where mycorrhizal symbioses become too costly for the host plant to maintain (i.e. plants no longer require N from the mycobiont), which could lead to loss of vital functional components of mycorrhizal communities. In addition, the planting of mycorrhizal seedlings establishes mycelial systems in the contaminated soils and allows gradual decontamination over time. Our results suggest that the greatest success may come from using indigenous species of host plants (grown in site soils) for initially establishing adapted mycorrhizal communities rather than inoculating plants grown in nursery soil mixtures with non-site-specific fungi.

In conclusion, this systems approach addressed fundamental questions in mycorrhizal ecology by considering PHC pollution as a form of environmental disturbance. Further research is needed for a more detailed understanding of interactions between PHCs and mycorrhizal systems in northern forest soils.

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Appendix A: ECM morphotype descriptions

ECM Morphotype	Gross Morphology	Mantle & Hartig Net	Emanating Hyphae	Rhizomorphs
Cenococcum	black, wooly to grainy, matte to shiny, unbranched, straight (1-2 mm)	OM regular synenchyma (stellate pattern), Hartig net present	brown-black hyphae (4-5 µm), sometimes copious, septate, no clamps, no ornamentation	none
MRA	black (hyaline at root tip), felty to wooly, matte, unbranched, straight (1-1.5 mm)	OM/IM felt prosenchyma, thin, Hartig net present	black or hyaline hyphae (2-3 µm), septate, no clamps, verrucose ornamentation	none
E-strain	orange-brown to dark brown (hyaline tip), grainy, matte, unbranched, straight (1-3 mm)	OM/IM net to non-interlocking irregular synenchyma, very thin, large cells (5-8 µm), Hartig net present	few hyaline hyphae (5-8 µm), septate, no clamps, verucose ornamentation	none
Amphinema	pale yellow, cottony, matte, dichotomous to irregular branching	OM net synenchyma, cell width 3-4 µm, Hartig net present	hyaline hyphae (2-3 µm), , septate, clamps, verrucose ornamentation	none
Piloderma	(not observed)	(not observed)	hyaline hyphae (2-3 µm), septate, clamps, verrucose ornamentation with needle-like crystals	pale yellow, loose, undifferentiated
Russulaceae 1	frosty orange-brown, smooth, grainy or velvety, matte, unbranched or dichotomous, straight or bent (1-3 mm)	OM/IM net to interlocking irregular synenchyma, small cell width (1-3 µm), Hartig net present	very few short hyaline hyphae (2-3 µm), septate, no (sometimes?) clamps, no ornamentation	none
Russulaceae 2 (*Lactarius*)	yellow-orange, smooth to velvety, matte, unbranched or dichotomous, straight to bent (1-3 mm)	OM non-interlocking irregular syenchyma, IM net synenchyma, variable cell width (4-8 µm), laticifers, Hartig net present	very few short hyaline to yellow cystidia (2-3 µm), slightly tapered out, no basal clamp, 100 µm long, septate, no clamps, verrucose or no ornamentation	none

ECM Morphotype	Gross Morphology	Mantle	Emanating Hyphae	Rhizomorphs
Russulaceae 3	creamy-yellow, smooth to velvety, matte, unbranched or monopodial pinnate, straight (1-4 mm)	OM net to non-interlocking irregular synenchyma, cell width 1-2 µm, Hartig net present	hyaline cystidia (2-3 µm), septate, no clamps, verrucose or no ornamentation	none
Rhizopogon-Suillus 1	rusty-brown, grainy to velvety, matte, coralloid (<1 mm), thick ropelike rhizomorphs and hyphal fans	OM/IM net synenchyma, Hartig net present, amorphous deposits on mantle surface	brown or hyaline hyphae (2-4 µm), septate, no clamps, verrucose or no ornamentation	brown-purple pigment (purple with KOH), restricted point attachment, slightly differentiated, septate, no clamps, amorphous globules on surface
Rhizopogon-Suillus 2	white, wooly, reflective (shiny), unbranched or dichotomous, bent to straight (2 mm), thick rope-like rhizomorphs	OM prosenchyma to net synenchyma, cell width 3-4 µm	hyaline hyphae (2-5 µm), septate, no (sometimes?) clamps, verrucose or no ornamentation	yellow-orange pigment (orange with KOH), slightly differentiated, septate, no clamps,
Thelephoraceae 1	black to dark brown, smooth, matte, unbranched, straight (1-3 mm)	OM non-interlocking irregular to regular synenchyma, Hartig net present	dark brown or hyaline hyphae (~2-3 µm), septate, clamps, no ornamentation	none
Thelephoraceae 2	gold-brown to orange-brown, smooth, matte, monopodial pinnate, straight (1-3 mm)	OM net synenchyma	long and copious hyaline hyphae (2-3 µm), septate, clamps	none
black	black (white at root tip), matte, dichotomous branching to coralloid (.5-2 mm)	OM net to non-interlocking irregular synenchyma, cell width ~4 µm, Hartig net present	black or hyaline hyphae (~3 µm), septate, no clamps	none
white 1	white, velvety, matte (1 mm)	OM net synenchyma	hyaline hyphae (1 µm), septate, no clamps	none
white 2	patchy white, grainy to wooly, reflective (~1 mm)	OM net synenchyma	hyaline hyphae (2-4 µm), septate, clamps	none

Appendix B: Photographs of field site, soils, bioassay, and ECM morphotypes

Plate 1: Soil Collection

1) Soil pit at the Kenneth Creek field site revealing organic, Ae, Bf, Bfj, Bm, BC, and C horizons of a Dystric Brunisol (Arocena and Sanborn, 1999); 2) Collecting the Bf layer; 3) Collecting the FH layer (background); 1x1 m^2 PHC-treated plot with the FH and Ae layers removed (foreground); 4) FHoil layer *in situ* 4 months after PHC treatment showing extensive yellow mycelia (a), small conifers (b), and herbaceous plants (c) growing within the plot; 5) Close-up of *Piloderma* mycelia from FHoil layer (a) and of a cluster of ECM root tips from the FH layer (b).

Plate 2: Plant-soil systems and ECMs

1) Bioassay: pine and birch systems following PHC treatment (a); lingonberry (b) and pine (c) single-plant systems in reconstructed soil layers; cleaned root systems of pine and birch at the 1-week sampling time (d); pine and lingonberry seedlings growing in CWD organic layer (e); ECM roots growing into the CWD substrate (f); 2) *Cenococcum* ECMs dominated the FH and FHoil layers in pine (a) and birch (b), PHC-treated (a) and untreated (b) systems; *Cenococcum* ECMs with profuse emanating hyphae (a,b) *Cenococcum* with very few hyphae (c); the typical stellate pattern of the outer mantle confirmed the identity of these ECMs (d); 3) E-strain ECM on pine; 4) Profuse emanating hyphae of *Amphinema* ECM on pine viewed using dissecting (a) and compound (b) microscopes; 5) Characteristic needle-like crystals ornamenting *Piloderma* hyphae were often found associated with pine root tips, although *Piloderma* ECMs were not observed in this study.

Plate 3: ECMs (cont)

1) Russulaceae 1 ECM on pine; 2) Russulaceae 3 ECM (center) and *MRA* (lower left) on birch roots; 3) Russulaceae 2 (*Lactarius*) ECM on pine (a); cluster of Russulaceae 2 tips growing into CWD (b); Russulaceae 2 ECMs often dominated the root systems of pine (c); 4) Development of *Rhizopogon-Suillus* 1 ECMs on pine roots from a small, whitish cluster (a), to a coralloid cluster with brown pigmentation and ropelike rhizomorphs (b), to a series of clusters with networks of rhizomorphs extending into the Bf soil matrix (c); 5) Cluster of *Rhizopogon-Suillus* 2 on pine roots showing extensive rhizomorph development; 6) Thelephoraceae 1 root tips (a), irregular to regular synenchyma pattern of outer mantle (b), and clamped hyphae (c); Thelephoraceae 2 ECM on pine.


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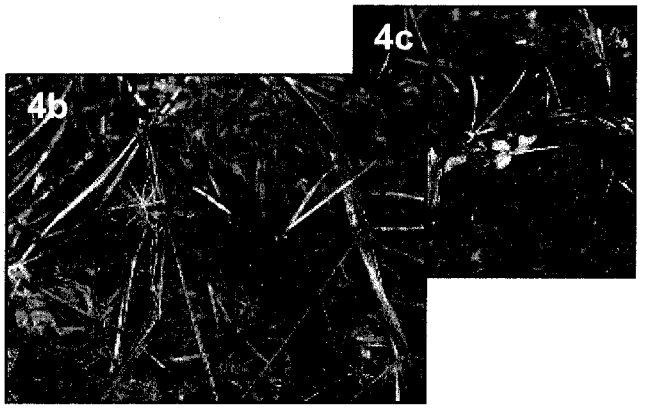
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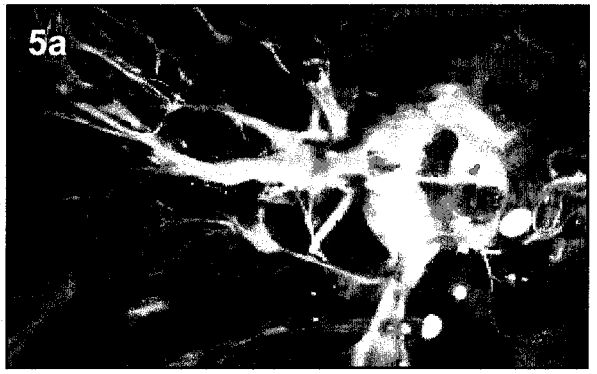
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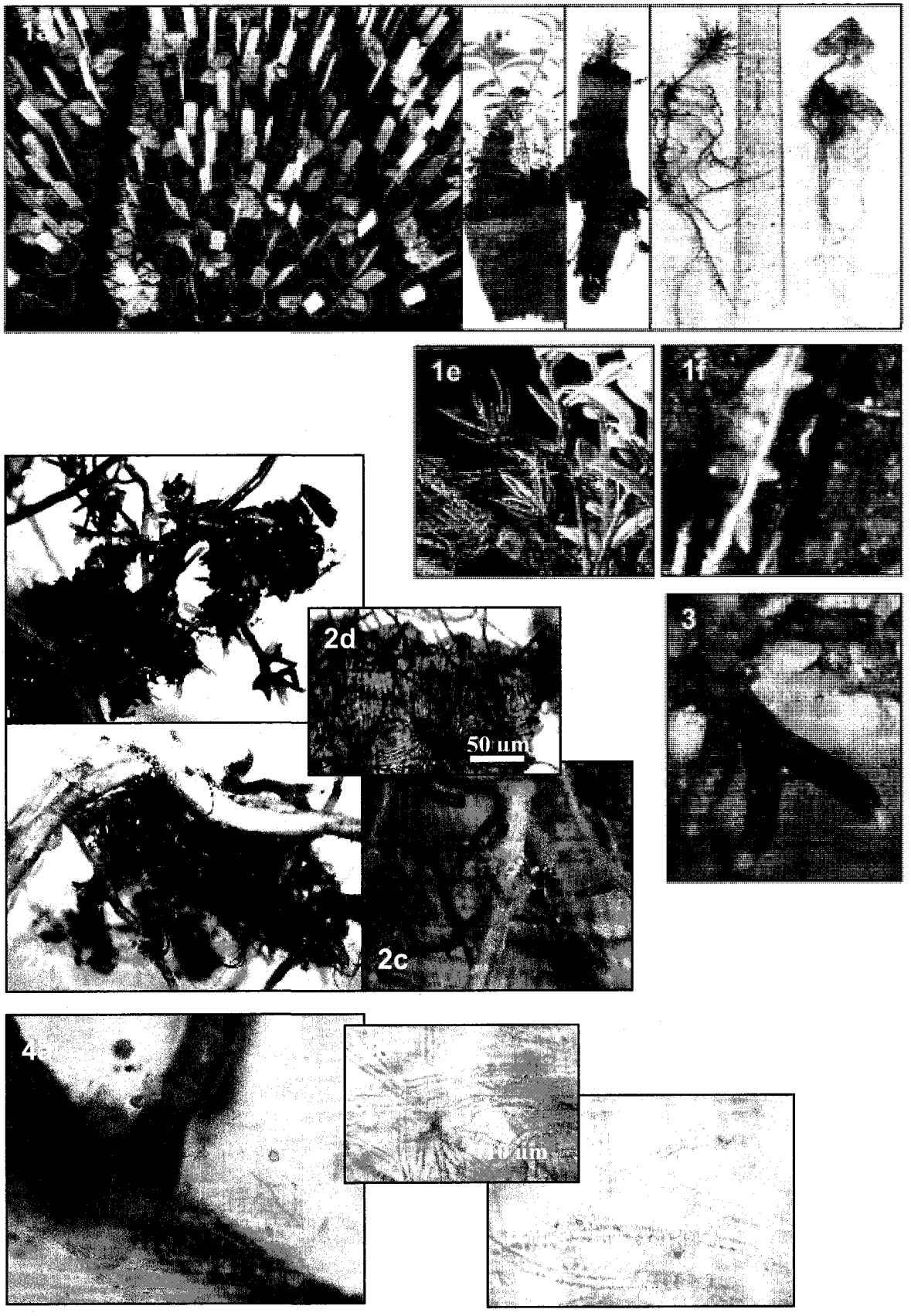
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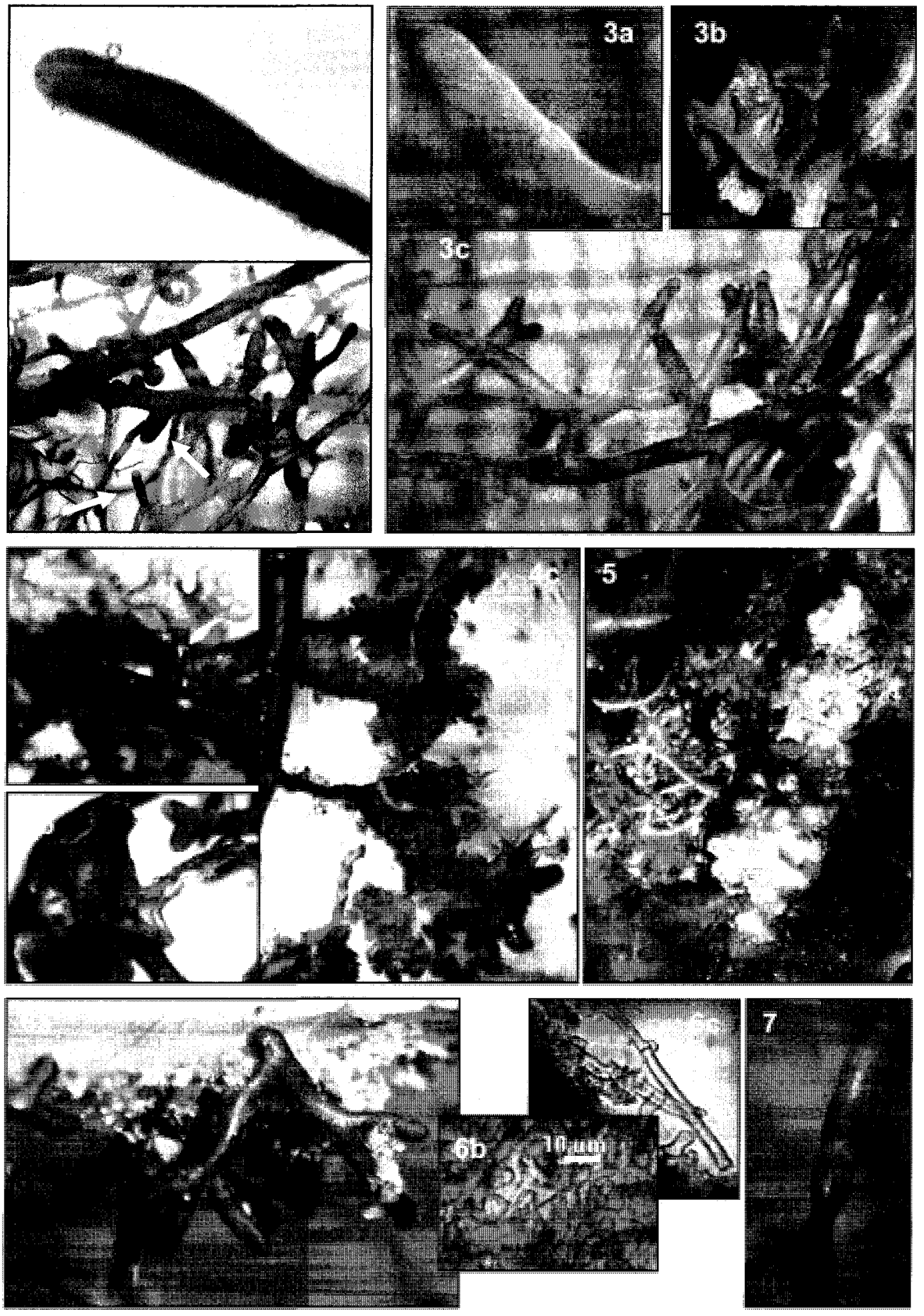
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